A STUDY OF AUTOGAMY IN THE CILIATE EUPLOTES MINUTA

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SUMMARY

1. Autogamy, or self-fertilisation in the marine ciliate Euplotes minuta has been studied by genetic and cytological means. The naturally occurring, between stock variation in four characters was used to contrast the genetic consequences of both conjugation and autogamy.

2. The existence of a number of different mating types allows crosses to be made, and the inheritance of the various characters to be analysed. All mating type alleles behave in crosses as if they are alternatives at a single locus, and as if there is a serial system of dominance whereby an allele assigned a number high in the series is dominant over all alleles lower in the series. Stock A25, previously described as being heterozygous at the mating type locus (Nobili and Luporini, 1967), was found to have the genotype mt^7/mt^7.

3. In this species, two types of stock are available; one type possesses the ability to pass through autogamy, the other type does not. The F_1 clones derived from crossing autogamous and non-autogamous parents were all capable of autogamy. The fact that the ratio of autogamous to non-autogamous clones was not significantly different from, firstly a 1 : 1 ratio in a backcross to one non-autogamous parent, and secondly a 3 : 1 ratio in an F_2 derived by crossing two F_1 clones, means that this trait could be controlled by a single locus, at which an allele which permits autogamy is dominant to an allele which does not allow this process to occur. Non-autogamous stocks must be homozygous for the non-autogamous alleles, and autogamous stock A25 must be homozygous for the allele which confers autogamy.

4. Antisera were prepared against two strains of E.minuta. When prepared

against an autogamous strain, the antiserum is efficient at immobilising the homologous and heterologous autogamous stocks, but gives a weak reaction against the non-autogamous stocks. An antiserum against a non-autogamous strain is also capable of discriminating between the two classes of stocks. Serotype was not inherited according to simple Mendelian rules. It is suggested that several loci may interact in the determination of this character.

5. By the use of electrophoresis of cell extracts, followed by detection of localised enzyme activity on a starch-gel, stocks were found to differ with respect to an esterase termed Es-1. This enzyme was characterised by inhibitor studies. Whereas the non-autogamous stocks used for genetic purposes possess an active form of Es-1, and are said to carry the allele $Es\text{-}1^{b}$, the autogamous stock A25 has no Es-1 activity and carries only the silent $Es\text{-}1^{a}$ alleles. The presence of esterase Es-1 is controlled by a single gene; the allele $Es\text{-}1^{b}$ being dominant to the silent allele $Es\text{-}1^{a}$. The results are consistent with the enzyme being a single polypeptide chain.

6. There was no genetic linkage between any of the four characters studied.

7. F_1 clones heterozygous at the loci controlling the four characters were passed through autogamy. Although after conjugation the segregation ratios for those traits which are certainly controlled by single genes, never deviated significantly from expectation, the phenotype of the F_1 was in most cases retained in the F_2 from autogamy. At the mating type and Es-1 loci the recessive homozygote appeared in only 1 in 30 exautogamous clones.

8. Nuclear events at conjugation and autogamy were studied by Feulgen and Giemsa staining. The micronucleus first divides mitotically and the two products undergo meiosis. Two of the 8 haploid products of the second meiotic

division persist and undergo a further division. Two of the four products are the gametic nuclei. After synkaryon formation there is normally one post-zygotic division; one product becomes the new micronucleus, the other enlarges into the macronuclear anlage. In the exconjugants this anlage differentiates to produce the sickle-shaped adult macronucleus. After autogamy the macronucleus rarely develops beyond the anlage stage; the anlage disintegrates and most animals which survive autogamy do so by regeneration of one or two fragments of the old macronucleus which are still present in the cell.

9. Knowledge of the sequence of nuclear events preceding conjugation and autogamy makes it possible to compute the expectations for the various genotypes after fertilisation. In P.aurelia, only 1 haploid nucleus survives beyond the second meiotic division, and produces gametes; therefore, exconjugants are alike, and exautogamous animals are homozygous. Due to the persistence, in E.minuta, of 2 haploid nuclei which appear to be the products of different meioses, gametic nuclei may be sisters, or non-sisters. Cytological data and genotypic ratios support the conclusion that in a cross Aa x aa the two exconjugants should be alike in 2/3 of the pairs, and unlike in 1/3. Crosses with E.vannus, E.crassus (Heckmann, 1963, 1964) and E.minuta fit this expectation. The expected ratio for synkarya after autogamy of animals of genotype Aa, is 1AA : 1Aa : 1aa. The genetic evidence shows that this expectation is clearly not fulfilled.

10. Macronuclear regeneration produces heterocaryons in other ciliates. The possibility that exautogamous E.minuta are heterocaryons is discussed. It is concluded that macronuclear regeneration is not an artefact of laboratory culture, but has an advantage over genuine autogamy in that maximum heterozygosity is retained. Autogamy starts a new phase of the life cycle in E.minuta;

animals initially being immature. The fact that autogamy can counteract the effects of senescence enables the nature of the rejuvenation process and theories of ageing to be reconsidered.

INTRODUCTION

The ciliated Protozoa exhibit considerable variety in the methods of reproduction which they employ. Even within a single genus there may be many permutations of the sexual process, and in addition alternative methods of counteracting the effects of ageing.

As early as 1889 Maupas stated that prolonged vegetative reproduction of ciliates was impossible. After a certain number of generations, senile degeneration set in. He believed conjugation, which occurs only after a definite period of immaturity, led to rejuvenation and a return to the normal rate of division. Thus according to Maupas the life cycle of ciliates is composed of regularly alternating periods of immaturity, maturity, and finally senility in the absence of conjugation. Although 80 years old these views are compatible with those of Sonneborn (1954a).

Faure-Fremiet (1953) associates the phenomenon of senescence, observed in the majority of ciliates, with the polyploidy of the macronucleus. During vegetative reproduction when the macronucleus divides amitotically the intactness of the genome may be gradually disturbed, and this would lead to malfunction of the macronucleus, and to senescence. The replacement of the macronucleus at conjugation corrects the physiological balance of the organism.

Certain ciliates have been cultured for many generations without any sexual process, and without any depression. The capacity of these ciliates for unlimited vegetative reproduction may be associated with the fact that in many of them there is a partial regulation of hyperploidy by means of extrusion of some nuclear material at division (see review by Raikov, 1969). The extrusion of chromatin from the macronucleus is most frequently observed in the family Colpodidae, and in Tetrahymena (Cleffmann, 1968), and many

holotrichs - just the ciliates in which indefinite vegetative growth is possible. This superficially lends considerable weight to Faure-Fremiet's argument.

Conjugation is not the only sexual process in ciliates which allows a replacement of the macronucleus. Autogamy, defined as internal self-fertilisation has been observed (but only sporadically) among the ciliate genera. It is reported as occuring in the following holotrichous ciliates: Tetrahymena rostrata during encystment (Corliss, 1952a,b,1956), Paramecium aurelia (Diller, 1934,1936; Sonneborn, 1939a), Paramecium calkinsi under rather specific conditions (Diller, 1948), Paramecium polycaryum (Diller, 1954), Paramecium jenningsi (Mitchell, 1962), and Frontonia leucas (Devi, 1961). The last 5 species undergo autogamy while in the free-swimming state. Among the spirotrichs, there is a report by Fermor (1913) of a process occurring in the cysts of Stylonychia pustulata which he interpreted as autogamy, although Hlowaisky (1926) could not confirm this. More recently this sexual process has been observed in Euplotes minuta (Nobili, 1966; Siegel and Heckmann, 1966). Among other members of the family Euplotidae there is an isolated case of autogamy in E.crassus (Nobili, unpublished); Katashima (1959a) also quotes Ito (unpublished) who finds it possible to induce autogamy in E.woodruffi by transferring the animals to dilute magnesium chloride solution.

In the early literature on this group of ciliates there are reports of endomixis-like processes involving a replacement of the macronucleus without meiosis, occurring during encystment. Möbius (1888) shows pictures of macronuclear breakdown in cysts of E.harpa; Klee (1926) observed macronuclear reorganisation in cysts of E.longipes. In the same organism she describes a reorganisation process in living animals. Similar claims were made by

Ivanič (1929a), who believed reorganisation of the macronucleus in free-swimming E.patella, and E.charon,to be endomixis or parthenogenesis. It cannot be excluded that in these cases the authors were looking at degenerating animals, or exconjugants in the case of the free-swimmers. Kimball (1939) says these early reports do not indicate a replacement of the macronucleus from micronuclei, and therefore do not suggest that they are observations of autogamy. In addition, autogamy has been induced experimentally by the split-pair method, and by abortive conjugation or "cytogamy", when one member of the pair fails to contribute to the fertilisation nucleus. These procedures have been employed in a variety of ciliates.

It is usually accepted that the nuclear events at autogamy, are identical to those at conjugation, differing only in that self-fertilisation occurs, and the new macronucleus arises from one of the products of division of the synkaryon. However this process has only adequately been studied genetically in P.aurelia, where the cytological events which produce genotypically identical exconjugants must, of necessity, result in homozygosity after autogamy.

Euplotes minuta was an attractive subject for a genetical study for a number of reasons. Taxonomically speaking it is a more complex ciliate than the much studied Paramecium aurelia and Tetrahymena pyriformis, which are themselves rather closely related, so we can see to what extent the models of nuclear behaviour and differentiation derived from these two ciliates may apply throughout the ciliate class. A series of mating types, and the appearance of autogamy, which is clearly visible in the living animal had already been described (Nobili, 1966; Siegel and Heckmann, 1966). In addition not all strains of E.minuta are autogamous; but the interbreeding of autogamous and non-autogamous stocks allows the inheritance of this trait

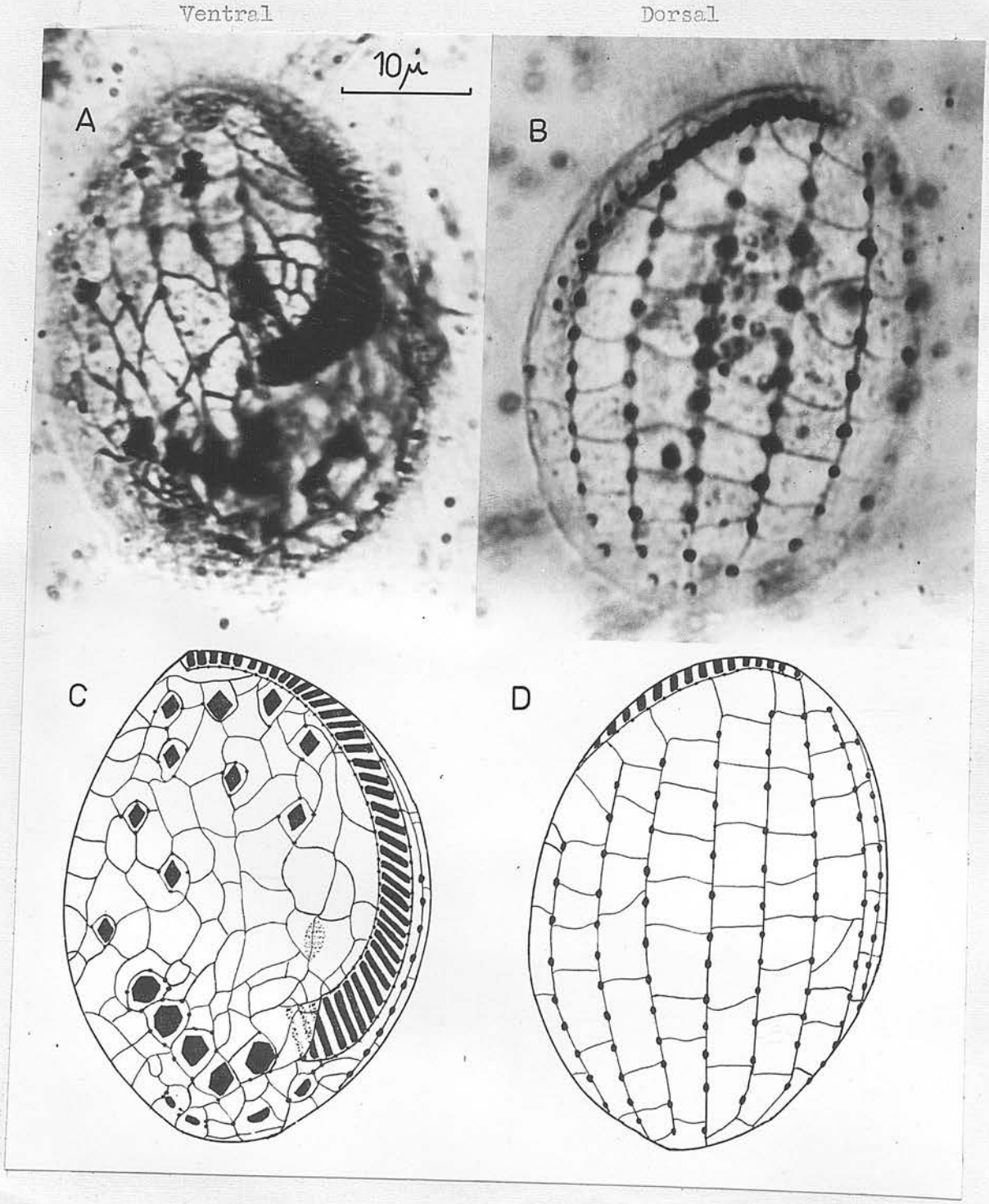
to be examined. A report by Nobili and Luporini (1967), studying mating type inheritance, presented a curious fact; namely that heterozygosity is preferentially maintained after autogamy, and only very rarely is there a segregation of the recessive homozygote. In preliminary studies of my own an attempt was made to induce mutations by exposing E.minuta to an X-ray dose of 5,000R, and subsequently isolating autogamous animals. Such a procedure in P.aurelia (Igarashi, 1966) results in more than 80% death, among the autogamous isolates. However I observed no increase in the death rate after autogamy, when compared with controls.

These two pieces of data suggest that autogamy has little or no effect upon the genetic constitution of this organism. These results needed corroboration using an independent, clearly genetically controlled trait, and a search was conducted for such a character using standard immunological and biochemical techniques.

This study combines genetical and cytological methods in an attempt to probe the exact nature of autogamy, and to throw some light on the advantage that autogamy confers upon the autogamous stocks. The findings will be discussed, together with the work of Heckmann, and Nobili and Luporini, on the same organism, and will be considered in the light of research upon this, and related genera.

Plate 1

Surface structure of Euplotes minuta from silver impregnated preparations. Diagrammatic representations from Heckmann and Frankel (1968).



MATERIAL AND METHODS

1. The Organism.

Euplotes minuta (Yocum) is a small marine hypotrichous ciliate. Originally reported in 1930, it has more recently been described by Borror (1962). The total body length is 48 to 65μ, averaging 57μ. The average width is 28μ. Ventrally, as can be seen in Plate 1, there are 10 fronto-ventral, 5 anal, and 4 caudal cirri, together with an adoral zone of membranelles (AZM). The dorsal surface bears short bristles, arranged in longitudinal rows, or kineties. Borror stated that there are always 9 such rows but a more detailed study by Heckmann and Frankel (1968) described variation from 8 to 11 kineties. The surface structures were observed from organisms impregnated with silver, following the Corliss modification (1953) of the method of Chatton and Lwoff (1936). Internally there is a large C or G shaped macronucleus, and one small micronucleus lying very close to the macronucleus.

Like most other members of the genus Euplotes, namely E.vannus (Heckmann, 1963), E.crassus (Heckmann, 1964), E.harpa (Katashima, 1952), E.cristatus (Wichterman, 1967), E.woodruffi (Ikeda, 1955; Rao, 1964) and E.patella (Kimball, 1939, 1942; Katashima, 1960, 1961), E.minuta expresses a system of multiple mating types. The exception is the fresh water species E.eurystomus (Katashima, 1959a) which has several syngens each with 2 complementary mating types.

The strains of Euplotes minuta used, and their sources are as follows. Strains VF17, A23, and 1F were collected at Villefranche (France), and strains A25, 12A, 22, A2, 11°, 16 and 20 at Leghorn (Italy). Stock A31 came from Venice (Italy) and strain PB from Portobello (Scotland). Stock 46C was derived by Nobili and Luporini (1967) and segregated from strain A25

after autogamy. Euplotes cristatus was collected at Aberlady (Scotland). I am grateful to Drs. Heckmann and Nobili for providing the stocks for this work.

2. Culturing Procedures

Euplotes minuta can be maintained in sterile sea water with the addition of Dunaliella sp. in "Erd-Schrieber" medium as a food source. The culture medium was essentially that used by Heckmann (1963) for E.vannus. Sea water was obtained from North Berwick, Scotland and Marina di Pisa, Italy. It was sterilized by heating to 80°C on three successive days.

Earth extract was prepared by the addition of 300 gm. of dry earth to 1l. of sea water. After boiling this mixture for 20 min. it was filtered and autoclaved at 15 lb./sq.in. for 15 min.

To 1l. of sterile sea water, 60ml. of earth extract, 80mg. of $NaNO_3$ and 35mg. Na_2HPO_4 were added, the pH of the mixture being about 8.0. 20ml. of concentrated Dunaliella suspension were then inoculated. The algae were allowed to grow at 18°C under a constant light source for 4 days before use.

Stock cultures of E.minuta were maintained in tubes at 18°C and were fed weekly. Cultures growing at maximum fission rates, or cultures under daily inspection, were grown in Boveri vials of 20ml. capacity. Single isolates were made from these. Single animals in the vegetative condition were isolated into dilute food in depression slides. They were permitted to grow at the maximum rate by serial reisolation every 3rd or 4th day.

The various phenotypes were determined from mass cultures. These were obtained by transferring the animals remaining after serial reisolation into test-tubes, and providing them with food for about 8 fissions. The volume and density of such cultures could be progressively increased by addition of concentrated food.

3. Isolation of autogamous animals.

One advantage of E.minuta is that living cells which are undergoing nuclear reorganisation can be easily recognised. Animals about to enter autogamy assume a characteristic "comma" shape prior to macronuclear breakdown; and at later stages the macronuclear anlage is easily visible as a clear ring in the centre of the organism.

Animals in autogamy were isolated into sterile sea water in depression slides and were left for 48 hours, or until reorganisation of the nuclear apparatus was complete. A little very dilute food was then added to the slides. After a further 2 days the slides were examined for exautogamous clones, which were then maintained by serial reisolation as described above.

4. Isolation of conjugating pairs.

Pair formation in E.minuta is not instantaneous, but appears to require the contact between animals of complementary mating type (Heckmann and Siegel, 1964). Pairs are not formed for 2 hours or more, depending on the actual stocks used, and the degree of starvation. The usual practice was to mix cultures of complementary mating type, which had just exhausted their food supply, and to leave these overnight. Like other members of this genus, E.minuta shows a preference for mating early in the morning (Katashima, 1959a; Wichterman, 1967). This procedure ensured firm pair formation by the following day.

Pairs were isolated into sterile sea water in depression slides. 24 hours later the slides were examined to ensure that conjugation had really occurred. Only those slides where there were two animals with clearly visible anlagen were retained. This controlled for cytogamy and premature separation of the pair. The two exconjugants were then reisolated into separate depressions, and treated in the same way as autogamous isolates. Animals reorganising after

autogamy and conjugation are extremely sensitive to environmental conditions. Attempts were made to reduce any changes in conditions by using the same batch of sea water both prior to, and after, isolation. In addition cultures of animals in Boveri vials were maintained in very dilute food in order to minimise the effect of removal of the food organism after isolation into depression slides. Despite these measures it was frequently found that all reorganising animals died, or the survival rate was much less than 1%. At each generation isolations had to be repeated, sometimes many times, until a survival rate of 3% or more was achieved.

5. Batch cultures.

Large cultures for zymograms were cultured at 25°C, and for injection, at 18°C, A tube or vial culture was first expanded to 250ml. in a sterile conical flask. When this had cleared - after 3 or 4 days - it was transferred to a sterile Thompson bottle, and the volume increased to 750ml. with algal suspension. The volume could be doubled every second day, but as the surface to volume ratio decreased, the density of the culture dropped.

6. Harvesting

When mass cultures had exhausted the food supply, they were filtered through 12 layers of muslin to remove algal debris. Animals which stuck to the cloth were washed off with sterile sea water. The culture was spun in tapered centrifuge tubes of 100ml. capacity in an oil-testing centrifuge at 250g. for 3 min. The supernatant was decanted leaving a loosely packed pellet. The animals were re-suspended in a few ml. of sterile sea water until harvesting was complete. The concentrates were pooled, and then spun in 10ml. tubes in a bench centrifuge at 400g. for 2 min. Generally 1 - 2 l. of

culture yielded 0.1ml. of clean packed cells.

When necessary cultures of algae were filtered and harvested in 10ml. tubes in a bench centrifuge operated at 700g. for 4 min.

7. Homogenization.

Cells were homogenized in glass tubes, using a power driven Tri-R teflon homogenizer. Microscopy showed that 30 strokes applied at speed 8 broke all the cells. Tubes were surrounded by tightly packed ice, and heating was reduced by allowing cooling for 30 sec. after every 10 strokes.

The cells were homogenized in the form of a wet pellet or were previously resuspended as described for the antigen extraction procedures. The whole operation was performed at 2 - 3°C. Homogenates were stored at -20°C.

8. Production of antisera.

Initially antibody production was stimulated in rabbits by subcutaneous injection of a homogenate in the form of a double emulsion. 0.2ml. of packed cells homogenized in 1ml. of 0.1M phosphate buffer pH 7.4 was used as antigen. By refractometry the protein concentration of this solution was 30 - 35mg./ml. The method of Herbert (1965) was used, as described by Sommerville (1967). 5 weeks later a test bleed was taken from the marginal ear vein. The blood was allowed to clot, the serum was decanted off and spun until free from erythrocytes. The serum was heated to 56°C for 30min. to inactivate complement, and was dialysed overnight at 2 - 3°C against several changes of sterile sea water.

By the criteria of immobilisation, and precipitation in agar the antibody titre was very low. Independent attempts were made to enhance antibody production by increasing the amount of soluble material present in the emulsion.

0.2ml. cells were homogenized in:-

a) 1ml. of 0.1M phosphate buffer pH 7.4 containing 0.2% sodium deoxycholate.

b) 1ml. of 0.1M phosphate buffer pH 7.4 containing 0.5% sodium deoxycholate.

c) 1ml. of distilled water.

Or d) 0.2ml. cells were ultra-sonicated for 9min., application being in 30sec. pulses interspersed with 1min. cooling.

No appreciable difference was detected in the titre of antisera produced against antigen injected subcutaneously after different extraction procedures.

It was assumed that the material injected was not available to antibody producing sites, and the method was therefore abandoned in favour of intravenous injection into the marginal ear vein.

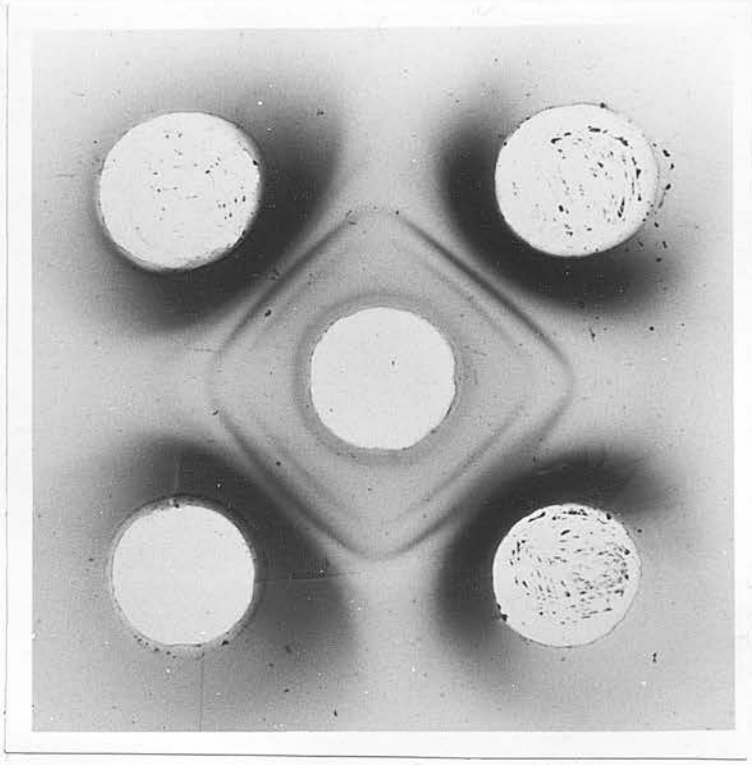
12 - 15l. of culture were harvested to give a pellet of 0.8ml. The washed pellet was resuspended in sterile sea water to give a volume of 8ml. After homogenization the extract was stored in 1ml. aliquots at -20°C. 1ml. of extract was injected twice weekly for 4 weeks. One week after the final injection serum was prepared, as described above, and was stored at -20°C.

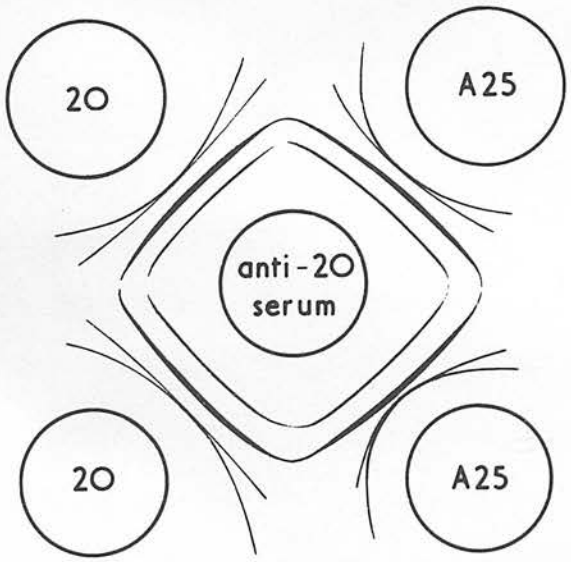
9. Immuno-diffusion Tests.

The presence of specific antibody in serum was demonstrated by the double diffusion method of Ouchterlony (1953). A 1% solution of Difco Noble agar in distilled water, containing thiomersal at a concentration of 0.05% was prepared in the usual way. 2.5ml. of warm agar were layered onto microscope slides pre-coated with a film of glycerol and agar. Wells of 3mm. diameter were aspirated 5mm. apart as shown in Figure 1. The central well was charged with antiserum and the outer wells with antigen in the form of a homogenate (see Crowle 1961).

Figure 1

Immuno-diffusion test showing the presence of specific antibody in serum. 3 precipitin bands were formed when anti-serum was tested against both homologous and heterologous antigens.





After incubation in a humidity chamber at room temperature (16 - 22°C) for 2 days, the gels were washed in 0.9% saline for 2 days to remove unprecipitated material, and dried under filter paper at 31°C. They were stained with a 0.1% solution of naphthalene black 12B (G.T.Gurr Ltd., London) in methanol, water and glacial acetic acid in the ratio 5:5:1, and differentiated in the same diluent.

From Figure 1 it can be seen that the homogenates contained at least 3 antigenic groups which both elicited antibody production, and were precipitated by the specific antibody-antigen interaction. It is not known which, if any, of these antigens corresponds to an immobilisation antigen. No differences between stocks other than those which could be attributed to differences in concentration of antigen, were detected using immunodiffusion tests. Reactions of identity were evident as fused precipitin arcs. These tests were used to investigate the efficiency of the different procedures for injection of antigen in stimulating antibody formation.

10. Immobilisation Tests.

Aliquots of serum were thawed at room temperature. A series of dilutions from 1/8 to 1/1024 of the serum in sea water were used to establish the similarity between all the different stocks, and to find the dilution which discriminated most efficiently between animals differing in serotype. 0.1ml. of diluted serum and 0.1m l. of culture fluid containing about 150 animals were placed in depression slides, and were incubated in moist containers at 18°C for 2 hours before scoring. Homologous animals were immobilised, or seriously retarded by the higher dilutions, whereas the effect of such serum on heterologous animals was very slight. Controls consisted of equal volumes of culture and sea water.

A final dilution of 1 part in 128 was found to be effective in immobilising homologous, but not heterologous animals. This "effective titre" was used in all subsequent tests. E.minuta was unaffected by normal rabbit serum even at a dilution of 1/2 indicating that the immobilisation reaction was indeed an immunological phenomenon.

11. Starch-gel Electrophoresis.

a) Preparation of the gel.

In order to obtain gels with reproducible properties it is important to standardise the method of preparation of the gel as much as possible.

Connaught hydrolysed starch (Connaught Medical Research Laboratories, Toronto, Canada) was used in all experiments, at an initial concentration of 9.4% w/v. Routinely 250ml. of starch suspension were heated in a 1l. Buchner flask. This gave sufficient volume for one gel plate 17.7cm. x 20.2cm. and 0.5cm. thick. The plate was prepared by sticking side-strips of glass onto a glass sheet using vacuum grease.

The Buchner flasks were heated with a regular swirling motion over a low flame. After the starch suspension had begun to boil, boiling was continued for 20 sec. Immediately after removal from the heat the flask was stoppered, and the side-arm connected to a vacuum pump. De-aeration was complete in about 35 sec. The gel was then poured into the glass mould, so that the liquid surface was just proud of the side-strips.

The gels were allowed to cool for at least 90 min. before use. After the first 30 min. they were transferred to a temperature of 2 - 3°C.

b) Application of Sample.

Homogenates were transferred directly from -20°C to 2 - 3°C. The

supernatant was absorbed onto small rectangles of 4MM Whatman Chromatography paper 2.5mm. x 8.0mm. Excess fluid was removed and the paper inserted into slots cut in the gel using a razor blade. 12 - 15 samples were applied to each gel.

c) Electrophoresis.

Electrophoresis was in the horizontal plane. Connection between the gel and the buffer in the electrode vessels was made by 6 thicknesses of 4MM. Whatman chromatography paper. The distance from wick to origin was 3cm. on the cathodal side. Separation was achieved at 300V and 70mA; the voltage was applied for $1\frac{3}{4}$ hours when esterases were being examined. Each gel was covered with a kyer of insulating polythene sheeting, and was cooled from both above and below by ice-cold water circulating through brass plates.

d) Electrophoresis Buffers.

The most satisfactory buffer system for esterase zymograms proved to be a phosphate/citrate buffer at pH 7.0. The electrode buffer was used at a concentration of 0.05M. 1 litre contained:-

2.06 gm. Trisodium citrate

3.2 gm. $NaH_2PO_4 . 2H_2O$

3.2 gm. Na_2HPO_4 (anhydrous)

The same buffer at a concentration of 0.01M was used to make the gels. The pH was checked and adjusted before use.

12. Staining of Gels.

a) Esterase assay.

After electrophoresis and removal of inserts, the gels were sliced horizontally with a sharp knife. The top slice was discarded as also were the sides of the gel - uneven migration and distortion of the bands can occur

in these regions. The bottom slice, which was 3mm. thick, was placed in 100ml. of the incubation medium.

Staining Buffer.

Sorensen's phosphate buffer pH 7.4

170ml. 0.1M KH_2PO_4

415ml. 0.2M Na_2HPO_4

415ml. distilled water.

Substrate.

Stock solution of 100mg. α-naphthyl propionate (Sigma Chemical Co., St.Louis, Missouri, U.S.A.) in 10ml. acetone.

Activators.

1% Triton-X100 (Sigma).

Sodium taurocholate (Koch-Light Laboratories Ltd., Colnbrook, England).

Dye Coupling Reagent.

Fast Blue RR salt (Diazotized product of 4-Benzylamino-2,5-dimethoxy Aniline-$ZnCl_2$) from Sigma.

1ml. of the stock solution of substrate was added to 5ml. of 1% Triton-X100 and 94ml. of phosphate buffer in which 539mg. of sodium taurocholate had been dissolved. Immediately before use 100mg. of dye were added. The mixture was shaken and poured over the gels. It was not necessary for all the dye to dissolve, but it was important to begin incubation without delay if excessive background staining was to be avoided.

The gels were stained for 3 hours at 30°C. During incubation esterases hydrolyse the propionyl ester with release of naphthol. This combines with the Fast Blue RR salt to produce an insoluble pigment at the site of the reaction. The positions of the enzymes, localised in the gel by electrophoresis, are therefore made visible as blue-grey bands. The gels were washed in running tap-water and were fixed in a mixture of methanol,

water and acetic acid in the ratio 5:5:1.

b) Inhibitor Studies.

The purpose of these studies was two-fold. Firstly to characterise the biochemical properties of the enzymes, and secondly to investigate the possibility of using these properties to distinguish between homozygous and heterozygous clones. When dealing with an absence-presence difference it is often possible to recognise heterozygotes because they have half the gene dose of the homozygote. By determining a concentration of inhibitor which will extinguish an enzyme band in a heterozygote, but not in a homozygote, one has a method of scoring the two genotypes.

For these studies the inhibitors were dissolved in the usual staining buffer and the gels were pre-incubated in this solution for 20min. at 30°C prior to staining. Staining was then conducted as usual, except that the incubation mixture contained the appropriate concentration of inhibitor. Eserine sulphate (Sigma) was used at concentrations of 10^{-3} M, 10^{-4} M and 10^{-5} M; p-hydroxymercuribenzoate (Sigma) at 10^{-4} M and 10^{-5} M. Diazinon (J.R.Geigy, Basle), an oily liquid, was first mixed with 2ml. ethanol before adding to the buffer. The final concentrations used were 5×10^{-4} M, 5×10^{-5} M and 5×10^{-6} M. The effect of heating the homogenate for 30min., at 60°C prior to electrophoresis was also examined.

c) Control without Substrate.

To demonstrate that the bands produced after incubation with the "esterase" stain are due to esterases and not to some non-specific component of the cell extract, as has been observed in the case of dehydrogenases (Shaw and Koen, 1968), a gel was split into two halves; one half was developed in the normal fashion, the other half was incubated in a staining solution from which the substrate - α-naphthyl propionate - had been omitted.

13. Cytological Staining Procedures.

a) Fixation.

Animals cultured in Boveri vials or 250ml. flasks were first filtered through 12 layers of cheesecloth, and then concentrated by gentle centrifugation at 250 g for 30sec. A few drops of the concentrated culture were placed on a microscope slide. These slides had been previously coated with a film of albumen made by the addition of 1 part of fresh egg albumen to 1 part of distilled water. After mixing, the albumen solution was filtered, and a few crystals of thymol added to the filtrate which could then be stored in the refrigerator. The albumen was allowed to air-dry on the slides for at least 24 hours before use.

Fixation with 45% acetic acid at room temperature for 5 min. was the usual procedure for Feulgen staining. Giemsa staining was preceded by fixation with Schaudinn's fluid prepared by the addition of 2 parts of saturated mercuric chloride to 1 part of 96% ethanol with 1 part of glacial acetic acid. This fixative was used at 55°C for 5 min.

An equal volume of fixative was added to the suspension of animals on the slide. During fixation the euplotes sank to the bottom of the drop, and excess fluid was removed. Subsequently the slide was covered with a dry coverslip which had been immersed in an emulsion of vaseline in ether. The slides were then 'squashed' by hand or in a slide press. It was hoped that squashing would break the nuclear membranes so making chromosomes visible. However the pressure exerted was insufficient to break membranes, but it did ensure even distribution of the organisms, which resulted in uniform uptake of stain.

After squashing, the slides were placed in 70% ethanol until the coverslips fell off spontaneously, leaving the euplotes embedded in the albumen.

The slides were then washed twice with distilled water, rinsed for 2 min. under running tap-water, and twice again in distilled water. The washed slides were subjected to 6 min. hydrolysis in 1N HCl at 60°C in order to destroy RNA. The washing procedure was repeated and the slides were then ready for staining.

b) The Feulgen Reaction.

The Schiff's reagent was prepared according to the method of Mackinnon and Hawes (1961). It was stored overnight in the dark at 4°C before use. Activated charcoal was not necessary for decolourisation. The crystalline basic fuchsin was obtained from G.T.Gurr Ltd. Slides were placed in the reagent for 2 hours, also in the dark, and were subsequently rinsed in 3 changes of freshly prepared sulphurous acid.

After washing for 12min. in running tap-water the slides were placed in 70% ethanol.

c) Giemsa Staining.

A concentrated solution of Giemsa (G.T.Gurr) was prepared by the method of Sonneborn (1950). 10ml. of this were added to 90ml. of 0.1M phosphate buffer pH 7.4 and slides were allowed to stain overnight. The stained slides were differentiated in 70% ethanol, the intensity of colour being controlled under the microscope.

Both sets of slides were dehydrated by passage through 70%, 80%, 96% and 2 changes of 100% ethanol; were equilibrated in a 1:1 mixture of 100% ethanol and xylol; were cleared in 2 changes of xylol and were finally mounted in DePeX (G.T.Gurr).

These staining procedures are a modified version of those employed by Kościuiszko (1965) in the study of P.aurelia.

14. X-irradiation of E.minuta.

The interautogamous period of strain A25, under the conditions of culture at 18°C, was determined as 21± 2 days (about 60 to 65 fissions). Exautogamous clones were established from single isolates of this stock, and were maintained for several weeks. 48 hours before the appearance of autogamy was anticipated, clones which had just exhausted the food supply were divided between 2 Petri dishes, 10ml. of culture being placed in each.

The animals in the experimental dish were irradiated with X-rays delivered by a Newton Victor Raymax 140 machine, operated at 140 kVp, and 5 mA. No filters were used. The dose given was 5,000R delivered at a rate of 787R/min. for 6.35 min. The dish was placed in a central position at a distance of 12.5cm. from the source. This was the maximum dose that could be given without heating. It was assumed that because of the uniform depth of the culture medium, and the mobility of the organisms, that they were all subjected to the same dose. The control dish was subjected to identical conditions without irradiation.

The 2 cultures were then given sufficient food for 9 fissions and were examined daily for the appearance of autogamous animals. When autogamy occurred, numerous autogamous animals were isolated from both experimental and control cultures. These were treated as already described and were scored after 1 week for survival.

15. Chemicals.

Unless otherwise stated all chemicals were of analytical reagent grade, and were obtained from British Drug Houses Ltd., Poole, England.

16. Photography.

Gels were photographed with overhead illumination using Kodak Panatomic X film with exposures of 1/60th of a second. The same film was used for black and white photography of permanently stained preparations using light optics and exposures of 1 to 7 seconds. Colour photography of stained organisms was performed with phase optics and Kodak High Speed Ektachrome film with exposures of 1/4 to $1\frac{1}{2}$ seconds.

Table 1

The effect of X-irradiation upon the survival rate of autogamous isolates of stock A25. The animals were irradiated with a dose of 5,000R as described in Material and Methods.

	Dead	Alive	% Survival
Irradiated	254	304	54.4
Unirradiated	269	267	49.8

$$\chi_1^2 = 0.49, \text{ p approx. } 0.5.$$

RESULTS

The results have been divided into four sections. Part I deals with the characteristics of the stocks, whilst Part II concerns the genetic analysis of between stock variation. The results presented in Part III are derived from cytological studies. In Part IV the theoretical genetic expectations after conjugation and autogamy, and their fulfillment will be reviewed.

PART I

Characteristics of the Stocks.

1. Autogamy.

Stocks A25, A23, A2, A31, PB and 46C will undergo autogamy. A period of approximately 50-70 fissions must elapse after autogamy, before these stocks are competent to either mate, or pass through autogamy for a second time. In this respect the autogamous stocks of E.minuta are unlike P.aurelia where there is no immature period following autogamy, although this phase is present in all exconjugant clones (Sonneborn, 1957). Luporini (1970) has studied the length of the immature period in several autogamous stocks, and found that the length of this period varies from strain to strain, but in general the ability to mate, and the ability to undergo autogamy appear simultaneously. The present work supports his findings. The percentage survival of autogamous isolates of stock A25 after irradiation was 54.4%, whereas 49.8% of the unirradiated controls survived autogamy (see Table 1.). Previous X-irradiation did not affect the death rate of autogamy (χ_1^2 = 0.49, p = approx. 0.5).

Stocks 12A, VF_{17}, 1F, 22, 11°, 16 and 20 have never been observed in autogamy.

Table 2

The mating types of the autogamous and non-autogamous stocks used in this study.

STOCK	MATING TYPE PHENOTYPE	MATING TYPE GENOTYPE	REFERENCE	AUTOGAMY
A25	VII	mt^7/mt^7	Present work	YES
"	VII	$mt^7/mt46C$	Nobili and Luporini 1967	YES
A23	VII	mt^7/mt^7	Nobili 1966 Heckmann and Frankel 1968	YES
A2	-	-	-	YES
A31	VII	mt^7/mt^1	Luporini and Nobili 1967b	YES
PB	-	-	-	YES
46C	46C	mt46C/mt46C	Nobili and Luporini 1967	YES
12A	VII	mt^6/mt^4	Luporini and Nobili 1967a	NO
VF_{17}	XI	mt^{11}/mt^1	Heckmann and Frankel 1968	NO
1F	XI	mt^{11}/mt^1	Luporini and Nobili 1967a	NO
22	III	mt^3/mt^2	Luporini and Nobili 1967a	NO
11^o	V	mt^5/mt^3	Luporini and Nobili 1967a	NO
16	not I,V, VII or XI		Present work	NO
20	I	mt^1/mt^1	Luporini and Nobili 1967a	NO

2. Mating Type.

The mating types of the stocks used in this study are summarised in Table 2. As already mentioned the present work was stimulated by Nobili and Luporini's work (1967) on the autogamous stock A25. When crossed with stock 20 (mt. I) carrying only the most recessive alleles, a 1:1 segregation of mating type was obtained in the F_1, indicating that stock A25 was heterozygous at the mating type locus. The recessive allele they called 46C. On repeating this cross the expected segregation of mating type was not observed. Subsequent work has confirmed that the sub-line of stock A25 used in this study is homozygous, and has the genotype mt^7/mt^7. Examination of the original line of A25 obtained from Nobili, usually showed a small number of mating pairs. This is consistent with the idea that animals of the genotype mt^{46C}/mt^{46C} were present. It may be assumed that animals of the genotype mt^7/mt^7 segregated at an equal frequency. If such animals have a slight selective advantage, or if severe sampling error is operating, a culture entirely of the genotype mt^7/mt^7 could have arisen. The possible source of this sub-line will be reconsidered later.

3. Serotype differences.

Ten stocks of E.minuta, and 1 stock of E.cristatus were tested against 8 dilutions of 2 anti-sera. One anti-serum was prepared against autogamous stock A25, the other against non-autogamous stock 20. The results are presented in Table 3, where + indicates immobilisation, ± severe retardation and - little or no effect in comparison with controls. The series shows firstly that there are strong cross reactions between stocks. Neither serum is stock specific, and the anti-A25 serum also retards the totally different species E.cristatus, at high concentrations. Secondly the sera

Table 3.

The reactions of 10 stocks of E. minuta and 1 stock of E. cristatus to 8 dilutions of 2 antisera. One antiserum was prepared against autogamous stock A25, the other against non-autogamous stock 20. These immobilisation tests were performed as described in Material and Methods, page 15.

Serum	Stock	Autogamous	Final serum dilution								Sea-water control
			1 in 8	1 in 16	1 in 32	1 in 64	1 in 128	1 in 256	1 in 512	1 in 1024	
Anti-A25 serum	A25	yes	+	+	+	+	+	+	+	+	-
	A23	yes	+	+	+	+	+	+	+	±	-
	A2	yes	+	+	+	+	±	±	-	-	-
	A31	yes	+	+	+	±	±	±	±	±	-
	46C	yes	+	+	+	+	+	+	+	±	-
	PB	yes	+	+	±	-	-	-	-	-	-
	20	no	+	+	±	-	-	-	-	-	-
	12A	no	+	+	±	-	-	-	-	-	-
	22	no	+	+	±	-	-	-	-	-	-
	1F	no	+	+	±	±	-	-	-	-	-
	E.cristatus	no	±	±	-	-	-	-	-	-	-
Anti-20 serum	A25	yes	+	+	+	±	-	-	-	-	-
	A23	yes	+	+	+	+	±	-	-	-	-
	A2	yes	+	+	±	±	±	-	-	-	-
	A31	yes	+	+	±	-	-	-	-	-	-
	46C	yes	+	+	+	±	-	-	-	-	-
	PB	yes	+	+	+	±	±	-	-	-	-
	20	no	+	+	+	+	+	+	+	+	-
	12A	no	+	+	+	+	+	±	-	-	-
	22	no	+	+	+	+	+	+	±	-	-
	1F	no	+	+	+	+	+	+	±	-	-
	E.cristatus	no	-	-	-	-	-	-	-	-	-

are not equally efficient in their reactions towards all the stocks. At concentrations of 1/128 and less, the anti-A25 serum reacts only towards the autogamous stocks, and at the 3 lowest concentrations the anti-20 serum reacts only with the non-autogamous stocks. It can therefore be concluded that the autogamous stocks have more antigens in common with one another than they share with the non-autogamous stocks. The reverse is likewise true. That the difference is one of quality, and not quantity of antigen produced, is suggested by the reciprocal nature of the results.

As already mentioned a dilution of 1/128 was selected for future testing of progeny. At this titre there is no cross reaction between autogamous and non-autogamous stocks when using anti-A25 serum, and when using the anti-20 serum the cross reaction is slight, and non-existent between the non-autogamous stocks and stock A25 used for this study.

The position of stock PB is equivocal. At the "effective titre" it is excluded from the autogamous group by the anti-A25 serum, and also from the non-autogamous group by the anti-20 serum. Although itself capable of autogamy, in at least one other character this stock resembles the non-autogamous stocks. It should also be mentioned that the origin of stock PB (Portobello, Scotland) is geographically isolated from that of all the other stocks of E.minuta.

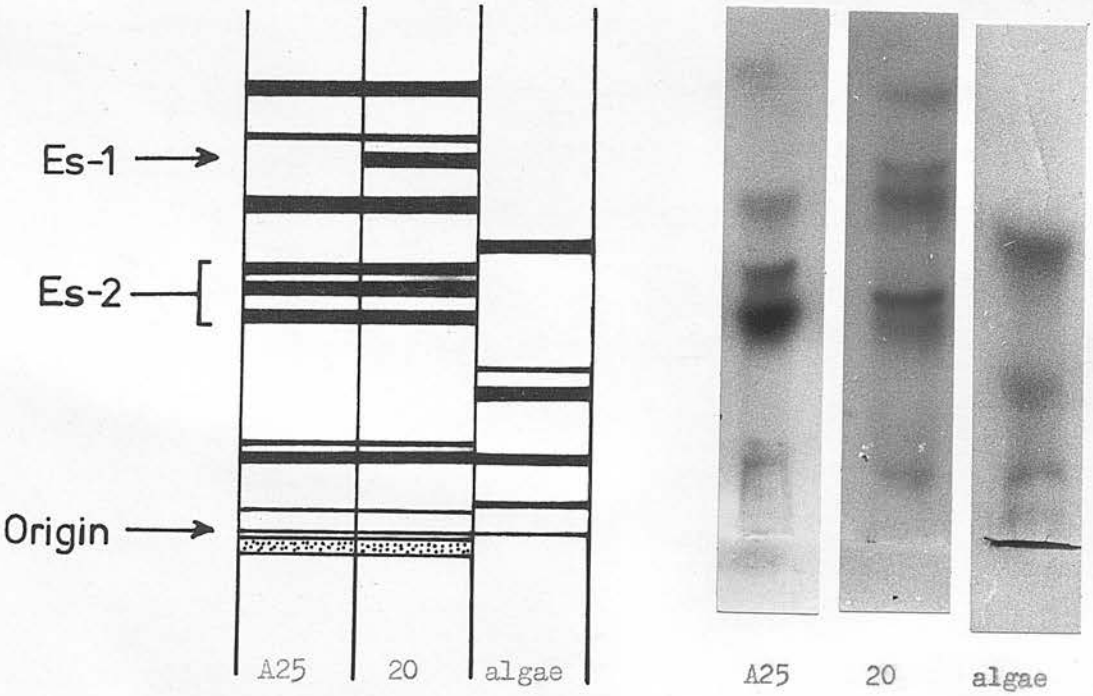
One further inference that can be drawn from the dilution series, is that the species Euplotes cristatus, has more in common with the non-autogamous stocks than with the autogamous ones.

4. Electrophoretic variation.

After starch-gel electrophoresis of fresh cell extracts unsuccessful

Figure 2

The esterase profiles of stocks A25 and 20 of E.minuta showing the difference at the Es-1 position. The alga, Dunaliella has no esterases which run to this position.



attempts were made to detect 6 phospho-gluconic acid dehydrogenase, hexokinase, glyceraldehyde 3-phosphodehydrogenase, alcohol dehydrogenase, fumarase, β-OH Butyric acid dehydrogenase, glucose-6-phosphate dehydrogenase, and glycerol phosphate dehydrogenase. Assays for NADP* linked isocitrate dehydrogenase, and NAD* dependent malic dehydrogenase, each showed two regions of activity on the gel. Glutamic dehydrogenase activity is associated with a single band. There are several acid, and several alkaline phosphatases present in homogenates of E.minuta, and a number of esterases. Among the stocks screened, variation was found only in one of the fast moving esterases.

Under the electrophoretic conditions used certain stocks were found to differ with respect to the presence or absence of this anodal esterase, termed Es-1. When present this enzyme will hydrolyse the esters α-naphthyl acetate, propionate, or butyrate, but superior resolution is obtained with α-naphthyl propionate. This ester was routinely used as the substrate. The remaining esterases are not so active towards this ester, under the conditions employed. The absence of any Es-1 activity could arise from two causes. The silent allele, which will be referred to as $Es\text{-}1^a$, may produce a protein lacking enzyme activity under the chemical conditions of the assay. Alternatively a deletion for this locus may be present in stocks without Es-1 activity, in which case no comparable protein will be present. Ruddle and Roderick (1968) were able to detect the enzyme product of a "silent allele" in the mouse, by altering the detection system. Changing the electrophoretic conditions in this study, produced such inferior separation of the esterases that the reason for the absence of esterase Es-1 activity must remain unsolved. Esterase Es-1 is not part of an isozyme system, as a single band varies, whilst the rest remain unchanged.

* NAD = β-Nicotinamide-adenine-dinucleotide.
NADP = β-Nicotinamide-adenine-dinucleotide phosphate.

Table 4

Esterase, Es-1 phenotypes, and genotypes if known, of 13 stocks of Euplotes minuta. Starch-gel zymograms were developed as described in Material and Methods, page 16

STOCK		PHENOTYPE		GENOTYPE IF KNOWN
		$Es\text{-}1^a$ (ABSENCE)	$Es\text{-}1^b$ (PRESENCE)	
A25	AUTOGAMOUS	+		$Es\text{-}1^a/Es\text{-}1^a$
46C		+		-
A23		+		-
A2		+		-
A31		+		-
PB			+	-
20	NON-AUTOGAMOUS		+	$Es\text{-}1^b/Es\text{-}1^b$
22			+	-
12A			+	-
VF_{17}			+	$Es\text{-}1^b/Es\text{-}1^a$
1F			+	$Es\text{-}1^b/Es\text{-}1^a$
16			+	-
11^o		+		-

The other most outstanding feature of the esterase profile, as shown in Figure.2 is the complex termed Es-2. 3 bands can be distinguished in this region. In repeat runs of the same stock intensity differences were experienced, such that the 3 bands may be of equal intensity, or one, usually the most anodal, or the central band is most pronounced. No mobility differences between stocks have been observed in this region.

The profile of esterases from an extract of algae showed no esterases with the mobility of Es-1 or the Es-2 complex. This is evidence that these enzymes are produced by the euplotes, and are not contamination products due to the presence of algae in homogenates. The genetic results confirm this view.

13 Stocks have been screened, and scored as $Es\text{-}1^{a}$ (absence of esterase activity) or $Es\text{-}1^{b}$ (presence of activity in the Es-1 position). The results are tabulated in Table 4. With 1 exception (stock PB) the autogamous stocks lack any enzyme activity in the Es-1 position, and with a further exception in stock 11^{o}, the non-autogamous stocks express the allele $Es\text{-}1^{b}$. One of the exceptions, stock PB, also differed from the other autogamous stocks with respect to serotype.

5. Biochemical Properties of Es-1 and Es-2.

One advantage of studying esterases was found to be that, with care, homogenates could be deep frozen for up to 2 weeks with no loss, or change, in enzyme activity. This observation was verified many times.

The effects of various other treatments upon the activity of $Es\text{-}1^{b}$ and the complex Es-2 appear in Table 5. This investigation was performed upon the pooled homogenates of 40 sublines of a clone heterozygous at the Es-1 locus: effects are therefore upon one dose of the active gene $Es\text{-}1^{b}$. It must be

Table 5

The effects of freezing, omission of substrate, heat, and esterase inhibitors upon the activity of Es-1^b, and the Es-2 complex. Zymograms were developed as described in Material and Methods, page 19

TREATMENT	INTENSITY OF Es-1^b	INTENSITY OF Es-2 COMPLEX
NORMAL INCUBATION PROCEDURE - FRESH HOMOGENATE	++++	++
NORMAL INCUBATION PROCEDURE - FROZEN HOMOGENATE	++++	++
OMISSION OF SUBSTRATE	-	-
HOMOGENATE HEATED TO 60°C FOR 30 MINS. PRIOR TO ELECTROPHORESIS	-	-
ESERINE SULPHATE 10^{-3}M	++++	++
ESERINE SULPHATE 10^{-4}M	++++	++
ESERINE SULPHATE 10^{-5}M	++++	++
DIAZINON 5×10^{-4}M	-	-
DIAZINON 5×10^{-5}M	-	+
DIAZINON 5×10^{-6}M	+	+
p-HYDROXYMERCURIBENZOATE 10^{-4}M	+	+
p-HYDROXYMERCURIBENZOATE 10^{-5}M	++	+

assumed that at least 2 genes are contributing to the intensity of the Es-2 complex, and maybe more.

Firstly it can be seen that the enzymes are heat labile, and that the enzyme reaction depends upon the presence of substrate. Secondly eserine sulphate has no effect upon the activity of either Es-1^b, or the Es-2 group. These esterases of E.minuta probably share little homology with the group 1 and 2 esterases in Tetrahymena pyriformis studied by Allen (1961; 1965; 1968). The esterase 1 isozymes in Tetrahymena are sensitive to eserine sulphate at a concentration of 10^{-4}M, and whereas the forms of esterase 2 in Tetrahymena are unaffected by eserine sulphate (10^{-2}M), they are inhibited by sodium taurocholate which was routinely included in the incubation mixture used for the assay of esterases in E.minuta. Thirdly both Es-1^b and Es-2 are inhibited by the organo-phosphorous compound Diazinon . The degree of inactivation increases with concentration, and the Es-2 complex is less profoundly affected.

The inhibition of these enzymes by Diazinon is a property shared by the so-called aliesterases, and cholinesterases. The inactivation arises because the compound reacts with the active site but the phosphoryl derivative formed is stable, and fails to break down. Inhibition by such a compound suggests that enzymic hydrolysis proceeds via an acyl-serine derivative, and there is thus a serine residue at the catalytic site of the enzyme (Myers, 1960). The insensitivity of the enzymes to eserine sulphate excludes them from the group of cholinesterases. Indeed, despite Allen's studies one might be surprised to find cholinesterases in Protozoa.

These enzymes are thus either simple ali-esterases or lipases - the term lipase being restricted to esterases attacking fatty acid esters with a long

carbon chain. They are unlikely to be true lipases as these do not attack esters with an aromatic residue such as the ∝-naphthol residue. Aliesterases are the main esterases in the plasmas of lower vertebrates (Augustinsson, 1961).

The mercury compound p-hydroxymercuribenzoate forms mercaptides with thiol (-SH) groups, and is a general enzyme inhibitor. Reactive-SH groups are thought to be involved in intramolecular linkages, and therefore contribute to the configuration of a protein (Cecil, 1963). The ability of p-hydroxy-mercuribenzoate to inhibit Es-1^b and the Es-2 complex suggests that these enzymes contain -SH groups. Secondly the efficiency of the inhibition gives some indication of the extent to which the configuration of the molecule is altered by the reaction. This in turn may be a reflection of the number of reactive -SH groups present in the enzyme. The results suggest that the Es-1^b enzyme is more susceptible to inhibition than the Es-2 enzymes. Presumably changes in shape in the former enzyme have a more profound effect upon the active site. There is very little evidence that -SH groups actually form part of the active site of esterases.

One further observation is that the 3 bands in the Es-2 complex behave similarly towards the various inhibitors; they have similar mobilities and substrate specificities. The fact that they share these biochemical properties means they qualify as isozymes, according to the definition of Markert (1968), and justifies considering them as a group. However until genetic evidence shows that a single gene difference affects all 3 bands concomitantly, further speculation as to the nature of the molecular relationship between the enzymes in the different bands is pointless.

6. Killing Activity

The ability of certain strains of Protozoa to kill other sensitive strains has been reported for P.aurelia (Sonneborn, 1959; Preer, 1968), P.bursaria (Chen, 1955), and P.polycaryum (Takayanagi and Hayashi, 1964). In 1966 Siegel and Heckmann described killing stocks of E.minuta, and in 1967 Heckmann et al. showed that the killing action of stock VF_{17} was associated with cytoplasmic particles. In 1968 (Heckmann and Frankel, 1968) stock VF_{17} was found to be a mate killer. When crossed to a sensitive strain the following result was obtained. "From two co-conjugants one normally developed into a healthy-looking exconjugant, whereas the other one failed to develop a large macronuclear anlage, became quiescent, and finally died." Both stock VF_{17}, and stock 1F (which was found to carry the same alleles at the mating type and Es-1 loci, as VF_{17}, and is probably in reality derived from VF_{17}) were examined for particles by the Feulgen technique. None were found. When crossed to stock A25 no evidence of the killing action of stock VF_{17} was observed. Both exconjugants formed large anlagen. No death occurred until the time when the anlage reorganises into a new macronucleus. Mortality is always high at this stage, but was no greater in crosses including VF_{17} than at any other time. In crosses with stocks 1F and A25, one exconjugant frequently failed to produce an anlage as will be shown in Part II of the results, this was not the A25 co-conjugant as would be expected if stock 1F was a mate killer.

Under the cultural conditions in this laboratory any killing particles must have been lost, or reduced to ineffective levels.

Table 6

The phenotypes of F_1, F_2 and backcross clones

Origin	% Survival	No. of viable clones	Mating type		Autogamy		Esterase		Serotype		
			VII	I	Autogamous	Non-Autogamous	Es-1^b	Es-1^a	A25-type	20--type	Un-classified
A25 x 20	65	100	99	(1)	99	(1)	8	0	21	70	8
F_1 (clone 71) x 20	10	40	22	18	27	13	-	-	6	31	2
F_2 from autogamy of F_1 (71)	3	21	21	0	21	0	19	1	9	2	10

Origin	% Survival	No. of viable clones	Mating type			Autogamy		Esterase		Serotype		
			VII	XI	I	Autogamous	Non-autogamous	Es-1^b	Es-1^a	A25-type	20-type	Un-classified
A25 x 1F	18	27	15	12	-	27	0	14	13	14	4	9
F_1 (clone 67) x 1F	10	71	17	24	10	22	26	41	12	8	16	26
F_1 (clone 24) x A25	25	107	41	47	-	106	(1)	0	46	50	5	45
F_1 (clone 24) x F_1 (clone 67)	7	60	12	15	-	24	10	24	23	15	6	39
F_2 from autogamy of F_1(24)	20	125	5	106	-	104	16	0	60	69	9	47

Origin	% Survival	No. of viable clones	Mating type			Autogamy		Esterase	
			VII	XI	I	Autogamous	Non-autogamous	Es-1^b	Es-1^a
A25 x VF_{17}	30	9	5	4	-	9	0	5	4
F_2 from autogamy of F_1 (7)	15	98	94	-	4	85	10	94	2

For technical reasons the number of clones scored for each character does not always equal the total number of viable progeny clones.

PART II

Genetic analysis of between stock variation

1. Cross 1.

a) A25 x 20

The primary aim of this cross was to investigate the inheritance of the difference in serotype between the 2 stocks. Information was also obtained concerning the mating type of stock A25, which was initially believed to be of the genotype mt^7/mt^{46C} (Nobili and Luporini, 1967). The mortality among the exconjugants was high and no attempt was made to recover pairs of clones (synclones) after the survival of both exconjugants. Instead approximately 150 pairs were isolated. After examination to ensure that true conjugation had occurred, survivors were allowed to reorganise and divide before single animals were reisolated to provide F_1 clones. These were tested for their mating reactivity towards stocks 20 (mating type I), A25 (mating type VII) and 46C (mating type 46C). Of the 100 F_1 clones, 99 were found to be of mating type VII. One clone expressed mating type I. With the exception of this one clone, all clones passed through autogamy within 3 months. The aberrant clone doubtless arose by cytogamy or separation of the conjugants, and was not the product of a cross.

The absence of any clones of mating type 46C allowed the deduction that the sub-line of stock A25 used for this cross was homozygous for the mt^7 allele. Immobilisation tests were conducted on the 99 clones at the age of approximately 30, 60 and 100 fissions. The final classification of the clones is given in Table 6. 70% of the clones had the serotype of the stock 20 parent. As all the other characters of the F_1 were those of the A25 parent, this was evidence that exchange of nuclei had occurred. 8 clones could not be classified as expressing the serotype exclusive to either parent. Of these 7 did not react

with either sera at the "effective titre", and 1 clone was immobilised by both sera. The simplest explanation for these clones was that the negative ones were not synthesising either antigen in sufficient quantities for immobilisation at the dilution used; and the doubly positive clone was producing both antigens at high levels.

In the majority of cases the serotype of the clone did not change with age. However 8 clones which reacted to neither serum at the age of 30 fissions were easily classified at 60 fissions. Six clones became determined for the 20 serotype, and 2 expressed the A25 serotype at the second testing. Heckmann and Frankel (1968) also observed a change in the surface corticotype of some F_1 clones between the ages of 13 and 70 fissions. It would appear that there may be a delay before the influence of incoming genes can be observed.

Although the parental stocks A25, and 20 were very uniform in their response to both antisera, there was variation within the F_1 clones. For example, when most members of a particular clone were immobilised by one antiserum, a small percentage of animals was sometimes unaffected. Similarly, in clones which were not immobilised by either serum to an appreciable extent, one could observe a few animals which were severely retarded by one, or both antisera.

This first cross was designed to give a segregation of mating type in the F_1, thereby allowing backcrosses to both parents to be made. However, due to the homozygosity of stock A25 at this locus, the F_1 were uniform for this character, and only one backcross was possible.

b) Backcross : F_1 (clone 71) x 20.

F_1 clone 71, with the A25 serotype, was backcrossed to stock 20. 40 viable clones were recovered. Of these, 22 showed the mating type VII, and 18 expressed

Table 7.

2 x 2 table showing independent assortment of the mating type and autogamy traits, in clones from the Backcross F_1 (clone 71) x 20.

	mating type VII	mating type I	totals
autogamous	16	11	27
non-autogamous	6	7	13
totals	22	18	40

χ^2_1 using Yates' correction = 4.23, $p < 0.05$.

mating type I. This 1:1 ratio (χ^2_1 with Yates' correction = 0.23, p approx. 0.6. See Snedecor, 1956, p.218) confirmed that clone 71 had the genotype mt^7/mt^1. The ability of these clones to undergo autogamy was tested; 13 were not observed in autogamy, despite regular examination over a period of 4 months, during which they were growing at a comparable rate to the 27 autogamous clones. 11 of the autogamous clones entered autogamy within one month of isolation and the remaining 16 passed into autogamy within 4 months. A ratio of 13:27 is significantly different from a 1:1 ratio at the 5% level of significance (χ^2_1 using Yates' correction = 4.23, $p < 0.05$), but in view of Heckmann and Frankel's results (1968) the suggestion that the ability to undergo autogamy is determined by a single gene cannot be ruled out.

Is the ability to undergo autogamy associated with mating type VII? Table 7 shows that there is no apparent linkage between the two characters (χ^2_1 using Yates' correction = 0.19, p approx. 0.7. See Snedecor, 1956, p.223). The possibility that autogamy could be controlled by cytoplasmic factors need not be entertained. The fact that all F_1s possessed the autogamy trait irrespective of their cytoplasmic origin (Heckmann and Frankel, 1968) showed that this character was not inherited in such a way.

When the progeny was classified for serotype, 31 clones expressed the 20-serotype, 6 the A25-serotype, and 2 remained unclassified.

c) F_2 from autogamy of F_1 (clone 71).

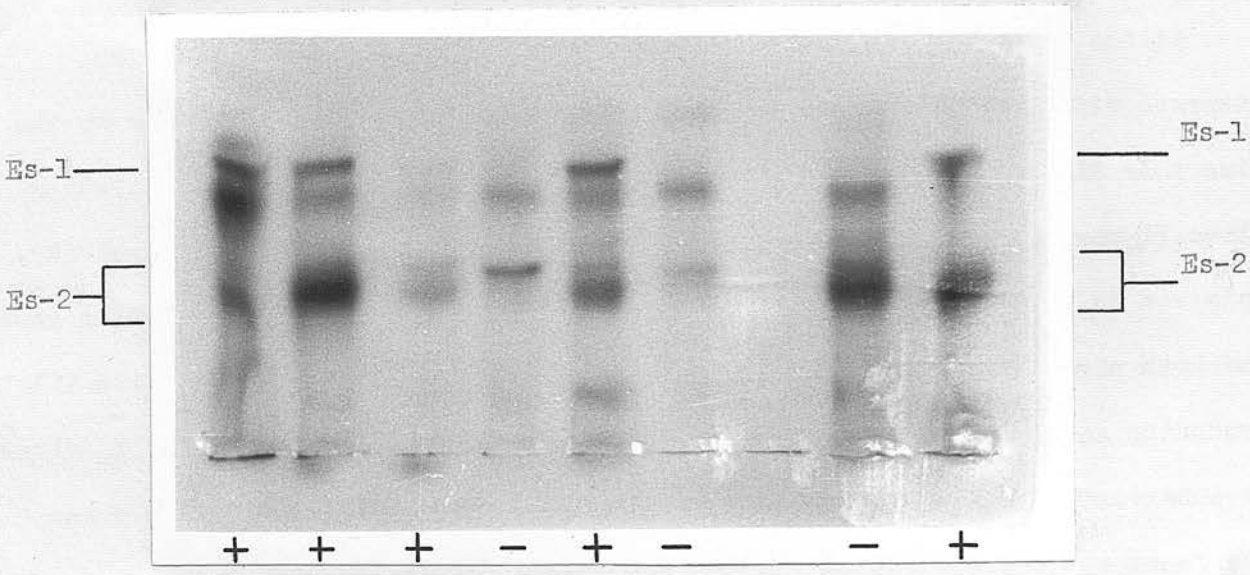
In order to investigate the effect of autogamy upon the segregation of the characters under study, many animals in autogamy were isolated from clone 71. Survival was about 3%. Many animals died before reorganisation of the macronucleus was complete. 21 exautogamous F_2 clones were obtained. All of these expressed mating type VII, as did clone 71; and all were autogamous. The expression of antigens was found to be very variable in these clones; 9 shared

the serotype of clone 71 and 10 were unclassified. Of these, 8 reacted with both antisera, and 2 with neither. Only 2 clones appeared to change serotype as a result of autogamy.

At the time when the original cross and the backcross were made, the esterase difference between stocks A25 and 20 had not yet been found. By the time this difference had been fully characterised only 8 F_1 clones (including clone 71) were being maintained. These were all found to have Es-1^b activity. This was to be expected if the enzyme was determined by a single Mendelian gene, and both parental stocks were homozygous. The result suggested that the allele producing an active enzyme was dominant over the "silent" allele. 20 F_2 clones were harvested and scored for the presence of Es-1^b activity. One of these was found to lack the active form of the enzyme. This one clone was unclassified serologically as it reacted with both antisera. The rarity of segregants in the F_2, regardless of the character considered will be discussed later.

In order to explore further the nature of autogamy in genetic terms, attention was directed to the Es-1 phenotype, rather than to the serotype. The inheritance of esterases in other organisms has often been found to be simple, whereas the results already described, using the serotype character suggest a complex inheritance of serotype with several loci and involved dominance relationships. It is unfortunate that the enzyme difference is of the absence/presence kind which does not permit identification of heterozygotes. However, there is as yet no character available in E.minuta which allows the heterozygotes and both homozygotes to be distinguished, without performing crosses.

Figure 3



Localisation of zones of esterase activity from 8 F_1 clones of a cross A25 x 1F.

+ indicates presence of esterase Es-1 activity.

- indicates absence of esterase Es-1 activity.

2. Cross 2.

a) A25 x 1F

When performing this cross many exconjugant pairs were discarded as it was found that only one member of the pair was forming a macronuclear anlage. The other exconjugant died shortly after separation. It was initially thought that this phenomenon could be due to mate-killing activity of stock 1F. 20 clones representing survivors from such crosses were grown up and tested for mating type. If they had been derived from the 1F parent after nuclear exchange they should express mating types VII and XI with equal frequency. However all 20 clones were found to express mating type VII, suggesting that they originated from the A25 conjugant, and were not the products of a true cross. The senescence of stock 1F was probably responsible for this phenomenon. 27 clones which were deemed to be the results of genuine crosses as both co-conjugants had developed as far as the anlage stage, were tested for the various phenotypes. The 15:12 ratio for segregation of mating type conformed with a 1:1 ratio (χ^2_1 using Yates' correction = 0.1, p approx. 0.75), and was expected if the genotype of stock A25 was mt^7/mt^7, and that of 1F was mt^{11}/mt^1. The 1:1 segregation of enzyme type was a surprise (χ^2_1 using Yates' correction = 0.0, p = 1.0). The previous F_1 had shown stock 20 to be homozygous for the Es-1^b allele, but stock 1F is apparently heterozygous. Figure 3 shows a photograph of one of the gels. The locus determining mating type is not genetically linked to the locus controlling the esterase phenotype (see Table 8). The two characters were inherited independently in the F_1 (χ^2_1 using Yates' correction = 0.05, p approx. 0.8). All 27 F_1 clones underwent autogamy within several months of isolation.

Stock 1F behaved in serotype tests very like stock 20, and therefore the anti-20 serum was used in testing the progeny of the A25x1F cross for serotype.

Table 8

2 x 2 Table showing independent assortment of the esterase Es-1, and mating type phenotypes in F_1 clones from the cross A25 x 1F.

phenotype	Es-1^b (presence)	Es-1^a (absence)	TOTALS
mating type VII	8	7	15
mating type XI	6	6	12
TOTALS	14	13	27

χ^2_1 using Yates' correction = 0.05
p approx. 0.8.

Table 9

2 x 3 table showing independent assortment of the serotype and mating type phenotypes, in F_1 clones from the cross A25 x 1F.

phenotype	A25-serotype	20-serotype	unclassified	TOTALS
mating type VII	7	4	4	15
mating type XI	7	0	5	12
TOTALS	14	4	9	27

From the G test $\chi^2_2 = 5.32$
$p > 0.05$.

The results from serotype analysis were also at variance with those obtained from the other F_1, as can be seen from Table 6. Although 9 clones were unclassified, there was a tendency to express the A25 serotype. Only 4 out of 27 clones were more completely immobilised by the anti-20 serum. That there was no apparent association between serotype and mating type is shown in Table 9. An RxC test of independence (the G test, Sokal and Rohlf, 1969, p.599) gave a probability of $p > 0.05$, indicating that the two characters were segregating independently.

As a result of the mating type segregation in the F_1 it was possible to make backcrosses to both parents. These 2 crosses, as well as the cross of two F_1 clones and the production of an F_2 from autogamy, were performed in Professor Nobili's laboratory in Pisa. When they were made I was not aware of the 1:1 segregation of the esterase Es-1 alleles in the F_1, and the choice of F_1 clones for the further crosses was most unfortunate in that they did not yield as much information as I had intended.

b) Backcross F_1 (clone 67) x 1F

An F_1 clone (67) subsequently shown to have Es-1^b activity was backcrossed to the 1F parent. A good 3:1 ratio for the Es-1^b: Es-1^a phenotypes was seen in the progeny (χ^2_1 using Yates' correction = 0.06, p approx. 0.8) as is shown in Table 6. This confirmed that both 1F and clone 67 had the genetic constitution Es-1^b/Es-1^a. On reaching maturity all progeny clones were tested for their ability to mate with stocks of mating types XI, VII and I. A 2:1:1 ratio for mating types XI:VII:I was expected when crossing an F_1 of genotype mt^7/mt^1 with mt^{11}/mt^1. The observed ratios of 24:17:10 were not significantly different from this expectation (χ^2_2 using Yates' correction = 2.24, p approx. 0.3). Out of 48 backcross clones, 22 were observed in autogamy within 4 months

Table 10

2 x 2 Table showing that there is no association between the ability to undergo autogamy, and a particular Es-1 allele, in clones from a back-cross F_1 (clone 67) x 1F.

phenotype	Es-1^b (presence)	Es-1^a (absence)	TOTALS
Autogamous	15	3	18
Non-autogamous	13	3	16
TOTALS	28	6	34

Fisher's exact test gives $p = 0.61$ that the two characters are inherited independently.

and 26 scored as non-autogamous. This was a good fit to a 1:1 ratio (χ^2_1 using Yates' correction = 0.20, p approx. 0.6). Of these 48 clones a random selection had also been examined for their enzyme phenotype. Table 10 demonstrates that there is no tendency for the ability to under autogamy to be associated with the Es-1^a phenotype, as would be expected if the loci determining these two characters were linked. Fisher's exact test (Sokal and Rohlf, 1969, p.593) gave a probability value of p = 0.61 indicating that these two characters are inherited independently.

F_1 clone 67 expressed serotype A25. 50% of the backcross clones could not be classified for serotype while 18 of these clones were immobilised by both antisera, 8 clones were not affected by either. As shown in Table 6, 8 clones expressed the A25 serotype and 16 the 20 or 1F serotype. 32 clones which had been characterised for serotype were also screened for their esterase profile. As is seen from Table 11 the two traits segregate independently. The G test for independence gave a probability of $p > 0.1$, showing no sifnificant association between these 2 characters.

Similarly, Table 12 shows that the serotype and the ability to enter autogamy are unassociated. The G test gave a probability of $p > 0.5$, showing no significant association.

c) Backcross F_1 (clone 24) x A25

F_1 clone 24 shown to have no esterase Es-1^b activity, was backcrossed to the autogamous parent A25. The survival from this cross was high - about 25% of exconjugants were recovered and 50 pairs of clones (synclones) were obtained. 23 such pairs were examined for esterase Es-1^b activity, but all 46 clones had none. This was compatible with the notion that both F_1 clone 24, and stock A25 were homozygous for the silent allele Es-1^a. The ratio of mating

Table 11

2 x 3 table showing lack of association between serotype and Es-1 alleles in clones from a backcross F_1 (clone 67) x 1F.

phenotype	A25-serotype	20-serotype	unclassified	TOTALS
$Es\text{-}1^2$ (absence)	0	2	3	5
$Es\text{-}1^b$ (presence)	6	10	11	27
TOTALS	6	12	14	32

From the G test $\chi^2_2 = 2.38$

$p > 0.1$.

Table 12

2 x 3 table showing that serotype and the ability to undergo autogamy are inherited independently in clones from a backcross F_1 (clone 67) x 1F.

phenotype	A25-serotype	20-serotype	unclassified	TOTALS
autogamous	3	7	11	21
Non-autogamous	5	7	12	24
TOTALS	8	14	23	45

From the G Test $\chi^2_2 = 0.35$

$p > 0.8$.

type VII to mating type XI of 41:47 fitted the 1:1 segregation (χ^2_1 using Yates' correction = 0.28, p approx. 0.6) expected if A25 was homozygous for the mt^7 allele, and clone 24 had the genotype mt^{11}/mt^7. Having paired clones made it possible to consider whether both exconjugants were alike with respect to genotype. In other words, are the two gamete nuclei in a conjugant, sister nuclei or can they be formed from different products of meiosis? 12 pairs were found in which both members shared mating type VII, 15 pairs shared mating type XI, and in 17 pairs one member was of mating type VII and the other of mating type XI. The significance of the ratio of pairs where the members show like genotype to pairs where the members differ in genotype will be discussed in Part IV of the Results. Only 1 clone out of 107 failed to enter autogamy. This one clone grew extremely slowly and finally died out. It is not impossible that it was amicronucleate.

Like clone 67, clone 24 also expressed the A25 serotype. 50% of the backcross clones also expressed this serotype, 5% the 1F or 20-type, and 45% were unclassified. Of this last group 23 clones were immobilised by both antisera and 22 were not immobilised at all at the "effective titre".

d) F_2 from cross of F_1 (clone 24) x F_1 (Clone 67)

Survival from this cross was only 7%. However 60 viable clones were obtained, although in only 1 case did both members of a pair survive. 47 clones were harvested and run on starch-gels. 24 possessed the active allele $Es\text{-}1^b$ and 23 had only the silent allele $Es\text{-}1^a$. This excellent 1:1 ratio is expected if the genotype of clone 24 was $Es\text{-}1^a/Es\text{-}1^a$, and that of clone 67 was $Es\text{-}1^b/Es\text{-}1^a$. The results from both backcrosses are consistent with this interpretation. With respect to the esterase alleles this cross was thus a repeat of the initial cross A25x1F.

The mating type alleles also segregated according to expectation yielding a ratio of 12:15, which did not differ significantly from a 1:1. (using Yates' correction = 0.1, p approx. 0.75). The backcrosses had again already confirmed the genotype of both F_1 clones.

Both F_1 clones were autogamous as previously mentioned, but a proportion of the F_2 progeny were scored as non-autogamous. The 24:10 ratio for autogamous:nonautogamous clones suggested that both F_1 clones were heterozygous at a locus which is largely responsible for the ability to undergo autogamy (the 24:10 ratio resembling a 3:1 ratio - χ^2_1 using Yates' correction = 0.16, p approx. 0.7).

As already stated, these two F_1 clones were serologically like stock A25. The F_2 clones remained largely unclassified, 19 being unaffected by the antisera, 20 being immobilised by both antisera. Only 15 clones resembled stock A25, and 6 the other parent.

e) <u>F_2 from autogamy of F_1 (clone 24)</u>

Cells in autogamy were isolated from clone 24. Survival was high (about 20%) and 125 viable clones were recovered. All of the 60 clones tested lacked the active esterase Es-1^b allele, as expected from the homozygosity of clone 24 for the esterase Es-1^a allele. This clone was known to be heterozygous at the mating type locus and yet only 5 recessive homozygotes, out of a total of 111 clones tested, appeared in the F_2. As will be seen in Part IV, the expectation is that 1/3 of the exautogamous clones, that is about 37, should be genetically mt^7/mt^7.

If indeed clone 24 was heterozygous for alleles controlling the ability to undergo autogamy, the same proportion of non-autogamous F_2 clones would be expected, but only 16 out of 120 were observed.

The majority (69) of F_2 clones retained the A25 serotype following autogamy, although 47 were unclassifiable. Only 9 apparently changed serotype. As this character is so ill-defined, and its mode of inheritance obscure, little stress can be placed upon this observation.

It is clear from the description of the studies on exautogamous clones, that a change in genotype does not usually occur after autogamy. However the only characters which are without doubt determined in a simple Mendelian fashion are mating type and the presence of esterase Es-1. Substantial data so far available related to mating type alone. In order to investigate the inheritance of both these characters simultaneously it was necessary to pass a clone heterozygous at both loci, through autogamy. As survival after autogamy decreases considerably with age a new cross had to be made. It was not possible to repeat the A25x1F cross, due to the senescence of stock 1F. Stock VF_{17}, bearing the same mating type alleles, was substituted for stock 1F.

3. Cross 3.

a) A25 x VF_{17}

No effort was made to obtain a large number of F_1 clones. With a survival rate of 30%, 9 viable clones were obtained; 4 of these expressed mating type XI and the other 5, mating type VII. There was also a 1:1 segregation for presence:absence of the allele $Es\text{-}1^b$. This confirmed that stock VF_{17} was like 1F with respect to these two loci, being heterozygous at both. Clone 9 selfed on achieving maturity, and this observation is discussed in a later section. 8 of the 9 clones entered autogamy within 4 months; one clone (clone 5) had not been seen in autogamy during this time, and was therefore classed as non-autogamous. As it was the only non-autogamous clone

observed out of a total of 135 F_1 clones, and as clone 5 shared the mating type and enzyme phenotypes of parent stock VF_{17}, it was conceivable that it had arisen by cytogamy, despite the measures taken to prevent retention of such progeny. Autogamy was finally observed in clone 5 fourteen months after isolation. In the interim no other reorganisation process such as selfing was ever seen. We must therefore bear in mind that any of the 75 clones derived throughout the genetic studies originally classed as non-autogamous might have been re-classified had they been examined regularly over a longer period of time. Delayed autogamy might explain the excess over Mendelian expectation, of non-autogamous clones in all crosses except the first back-cross.

b) F_2 from autogamy of F_1 (clone 7)

An F_1 clone carrying the following alleles:- mt^7/mt^1 and Es-1^b/Es-1^a was used to generate an F_2 from autogamy. Survival was about 15% and 98 new F_2 clones were produced. 4 of these expressed the recessive mating type I, and a further 2 lacked Es-1^b activity. All the remaining clones showed the phenotypes of the parent. It had been hoped that this number of clones might be sufficient to demonstrate that a change in genotype for one locus was associated with a change in genotype at another locus, more often than by chance alone. However the 4 F_2 clones which expressed mating type I did not include either of the clones which were now homozygous for Es-1^a. 10 out of 95 of these clones did not go into autogamy within the 4 month period in which they were examined for this trait. With respect to the other two loci, these 10 clones retained the phenotype of F_1 clone 7.

When these F_2 clones were first tested for mating type the majority of clones were unreactive; only 4 clones reacted at the first testing. 3 of

these had a reduced fission rate and were subsequently found by Feulgen staining to be amicronucleate. It would appear that amicronucleate clones have a short immature period in E.minuta.

4. Summary of the Genetic results

a) Inheritance of mating type

All alleles behaved in crosses as if they were alternatives at a single locus, and as if there were a system of dominance whereby an allele high in the series was dominant over all alleles lower in the series. Stock A25 was shown to have the genotype mt^7/mt^7 and stocks 1F and VF_{17} the genotype mt^{11}/mt^1. After autogamy of heterozygous clones, only 9 out of a total of 230 exautogamous clones (1 in 26) expressed the phenotype of the recessive allele. Usually there was no change in mating type following autogamy, although a period of immaturity comparable to that following conjugation was observed for all clones, with the possible exception of those which were amicronucleate.

b) Inheritance of Esterase Es-1

The presence of this esterase appeared to be controlled by a single gene-termed $Es\text{-}1^b$ - which was present in the homozygous condition in stock 20, and in the heterozygous condition in stocks 1F and VF_{17}. The alternative silent allele $Es\text{-}1^a$ was characteristic of most autogamous stocks. The active allele was dominant to the silent one. Such a situation has also been reported for esterases in the Sulphur Butterfly, Colias (Burns and Johnson, 1967), Mus (Pelzer, 1965), Peromyscus (Randerson, 1965) and Microtus (Semeonoff and Robertson, 1968) to quote just a few examples.

The alternative explanation that the expression of Es-1^b was regulated by controlling or suppressor genes could not be excluded. In vitro mixtures of total cell extracts lacking Es-1 activity with those which possess activity, produced bands on starch-gels, so that such control could not operate at the protein level.

Whether the null alleles present in all stocks were the same is unknown. No complementation was observed in crosses involving silent alleles from different sources, but such intra-allelic complementation would only be expected if the enzyme was a polymer.

The simplest explanation is that the esterase Es-1 is a protein consisting of a single polypeptide chain, or a homopolymer consisting of several identical chains, specified by a single locus. Until stocks possessing Es-1 esterases of differing mobilities are available, it is difficult to assess the degree of polymerisation.

The ratios from crosses fit very closely those expected on the basis of this hypothesis. There appears to be no significant selective advantage of the active esterase allele Es-1^b over the silent allele Es-1^a (at least in terms of growth rate). In addition the esterase locus is clearly unlinked to the locus determining mating type, or loci controlling the other characters studied.

Autogamy of clones heterozygous for the esterase alleles showed that exautogamous clones homozygous for the Es-1^a allele segregated in only 3 out of 116 cases.

c) Inheritance of the Autogamy trait

The ability to undergo autogamy may be controlled by a single locus at which an allele which permits autogamy is dominant to one which does not allow

this sexual process to occur. Non-autogamous stocks must then be homozygous for the non-autogamous alleles, and the competence to undergo autogamy of all F_1 clones derived from crosses with stock A25, implies that this stock is homozygous for the allele which confers autogamy.

The fact that the ratios of autogamous to non-autogamous clones are not significantly different from firstly, a 1:1 ratio in the backcross to the non-autogamous parent 1F, and secondly a 3:1 ratio in the F_2 derived by crossing two F_1 clones, merely means that this trait could have a simple genetic basis, but it does not prove that this is the case. In the other backcross to non-autogamous parent 20, there was an excess of autogamous clones, the ratio being significantly different from a 1:1 ratio, but only at the 5% level.

If we assume that a single locus controls the autogamy trait, we must accept that its expression is modified by other factors. F_1 clones usually enter autogamy one month after isolation, but considerable variation is observed in the length of the inter-autogamous period in animals derived from backcrosses and in the F_2. Clones which are scored as non-autogamous at the end of the 4 month inspection period might have to be reclassified if they were examined for longer. With the exception of the backcross to stock 20, there is a slight excess of non-autogamous clones over expectation on a Mendelian basis. The excess clones may have a longer interautogamous period.

Variation of another kind is also observed in clones which can enter autogamy. Although the process may be initiated it is not always completed. Thus in some clones macronuclear breakdown and micronuclear division occur, but a new macronuclear anlage is not formed; such clones die when autogamy occurs. In other clones an anlage is formed, but then the reorganisation process stops. These clones also die on entering autogamy. It is clear that the genetic background can influence both the frequency with which

Table 13

Serotype classification of F_1, F_2 and backcross clones based on repeat immobilisation tests, performed as described in Material and Methods, page 15.

	Origin	Total clones tested	A25-serotype	20-serotype	Unclassified		Total Unclassified
					-ve to both sera	+ve to both sera	
Cross 1	A25 x 20	99	21	70	7	1	8
Cross 2	A25 x 1F	27	14	4	6	3	9
Backcross 1	F_1 (clone 71) x 20	39	6	31	1	1	2
Backcross 2	F_1 (clone 24) x A25	100	50	5	22	23	45
Backcross 3	F_1 (clone 67) x 1F	50	8	16	8	18	26
	F_1(clone 24) x F_1(clone 67)	60	15	6	19	20	39
	F_2 from autogamy of F_1 (clone 71)	21	9	2	2	8	10
	F_2 from autogamy of F_1 (clone 24)	125	69	9	34	13	47

autogamy occurs, and also its success.

So far no linkage has been demonstrated between the autogamy trait and any other character. Following autogamy of clones (which we will presume are heterozygous for a major gene determining the ability to undergo autogamy), in only 26 out of 236 cases did clones incapable of autogamy arise.

d) Inheritance of antigenic type

In this instance it is not easy to translate the phenotype, namely the ability of an animal to be immobilised by a particular antiserum known from immunodiffusion tests to be multivalent, into genetic terms. This may be because this character is not a simple one, but is the outcome of the interplay of many loci. This would be the case if the antigenwas a large molecule composed of several different polypeptide chains, each of which may be capable of eliciting an antibody response. Finger et al. (1966) have obtained antisera which contain antibodies against determinants which they believed to be situated on different subunits of the Paramecium immobilisation antigen.

Alternatively, the antigenic difference picked up in the immobilisation test may be a simple one controlled ultimately by a single gene. In this case the mechanisms whereby the expression of this gene can be affected must be most complex, and may be influenced by environmental factors such as temperature, growth rate, and the genetic background. Table 13 contains the full data from immobilisation tests.

There are no grounds for assuming that the genes which control the expression of the surface antigens in the autogamous stocks are allelic with those which produce the serotype characteristic of the non-autogamous stocks. In Paramecium aurelia all stocks of syngen 1 can express several antigens, but only one is expressed at a time. The genes controlling the different antigenic types are non-allelic and unlinked. In crosses where the allelic

antigenic types of the parents can be distinguished serologically (as in the case of 60G x 90G) the F_1 is immobilised by both antisera, but the relative proportions of the two antigens in the F_1 may vary (Beale, 1954).

Any complete explanation of the situation in E.minuta must explain the fact that on crossing stock A25 to stock 20, the progeny show predominantly the serotype of stock 20, but on mating stocks A25 and 1F, most F_1 clones express the serotype of the A25 parent. This observation could suggest dominance among a series of alternative alleles (as in the multiple mating type alleles) in which the 20 serotype is dominant to the A25 serotype, which in turn is dominant to the 1F serotype. The fact that it was not possible to distinguish between stocks 20 and 1F serologically does not necessarily mean that the antigens are identical in all respects in these stocks.

However it is quite clear that dominance is not complete, as both parental classes do appear in the F_1. If the serotype is determined by a single locus it is necessary to invoke variation in gene expression among the progeny, as occurs in Paramecium. In heterozygotes the serotype expressed might depend upon the presence of other genes. This is just another way of stating that dominance can be modified. If we accept this, we must also concede that in the A25 x 20 cross modifiers in stock 20 favour the expression of the 20 serotype, whereas in the A25 x 1F cross the modifiers will favour the expression of the A25 serotype in the F_1 and suppress the expression of the non-autogamous antigens.

If we assume that each parent stock is homozygous for the major gene determining its antigenic type not only should the F_1 be genetically uniform, but only two genotypes should appear in each set of backcross progeny. At least 50% of backcross clones should resemble the parent used while the remaining 50% should be heterozygous, and in these animals the expression of serotype may depend upon the modifiers which are segregating. Backcrosses 1

and 3 are consistent with this hypothesis but the backcross to 1F is not. In this cross there is a deficiency of clones showing the non-autogamous serotype. We must propose that modifiers from stock 1F suppress this serotype, and we must allow that a proportion of the unclassified clones are homozygous for the 1F serotype. Similarly in the F_2 clones produced by crossing two F_1s there is a further deficiency of 1F or 20 offspring, possibly for the same reason.

Let us consider another alternative. Suppose that two non-exclusive, unlinked loci are responsible for the differences between the autogamous and non-autogamous stocks. Autogamous stock A25 would then carry two active alleles at a locus determining the production of the A25 antigen, but two non-active alleles at a locus responsible for the non-autogamous serotype (e.g. A^+/A^+, B^-/B^-); in stocks 1F and 20 the position would be reversed (A^-/A^-,B^+/B^+). Like the single locus model the F_1s should all be alike (A^+/A^-,B^+/B^-) and one must again resort to differences in genetic background to explain the results. In backcrosses, four instead of two genotypes of progeny should appear. (From the F_1 x 20 backcross the genotypes would be:- A^+/A^+,B^-/B^-; A^+/A^-,B^-/B^-; A^+/A^+,B^+/B^-; A^+/A^-, B^+/B^-.) In all three backcrosses 50% of clones will carry active alleles for one antigen only. However, one half of these will bear only one dose of the active allele, so that any genes tending to suppress the non-autogamous phenotype would have more scope to produce an effect.

On this hypothesis the F_2, derived by crossing F_1 clones 67 and 24, should show a phenotypic ratio of 9 clones carrying both active alleles (either in one or two doses), to 3 carrying the active A25 allele only, to 3 bearing only the active 1F or 20-serotype allele, to 1 clone with inactive alleles at both loci. If the unclassified clones include those bearing active alleles at neither or

both loci, the 15:6:39 ratio observed (see Table 13) fits reasonably well to a 3:3:10 ratio ($\chi^2_2 = 3.90, p > 0.1$), although there is still the deficiency of the non-autogamous serotype. The increase in variability in the F_2 generation, seen in the larger number of unclassifiable clones, also suggests that more than one locus may be responsible for determining this character.

As yet no completely satisfactory genetic explanation of the inheritance of serotype is available. In order for either of the two simple models just considered, to fit the data, modifying genes had to be introduced. In P.aurelia and T.pyriformis it is well known that the "cytoplasmic state" of the cell is important in determining which of several loci is expressed. It is also apparent from the work of Finger et al., (1966) that the two alleles in a heterozygote are not equally expressed in every clone of P.aurelia. Although there is no precedence for allele preference in E.minuta it is possible that other genes present, as well as the recent history of the cytoplasm, may influence the expression of serotype.

The rarity with which there is a definite change in serotype following autogamy, from the autogamous to the non-autogamous type, is in accordance with the behaviour of the three other traits. The appearance of unclassifiable clones after autogamy may again suggest that the expression of genes in heterozygotes may be modified.

5. Intraclonal Variation

Several situations are known in which ciliates of identical genotype differ in phenotype. E.minuta supplies a further example. Age-dependent intraclonal conjugation has been reported by Heckmann (1964, 1967) in E.crassus and Nobili and Luporini (1966) have cited a case of conjugation in a young clone of E.minuta

Table 14

Selfing behaviour of paired clones of *E.minuta* derived by splitting a selfing pair while in the very early stages of conjugation.

Clone title	Presence of pairs
1A	+
1B	-
2A	-
2B	-
3A	+
3B	+
4A	+
4B	+
5A	+
5B	-
6A	-
6B	+

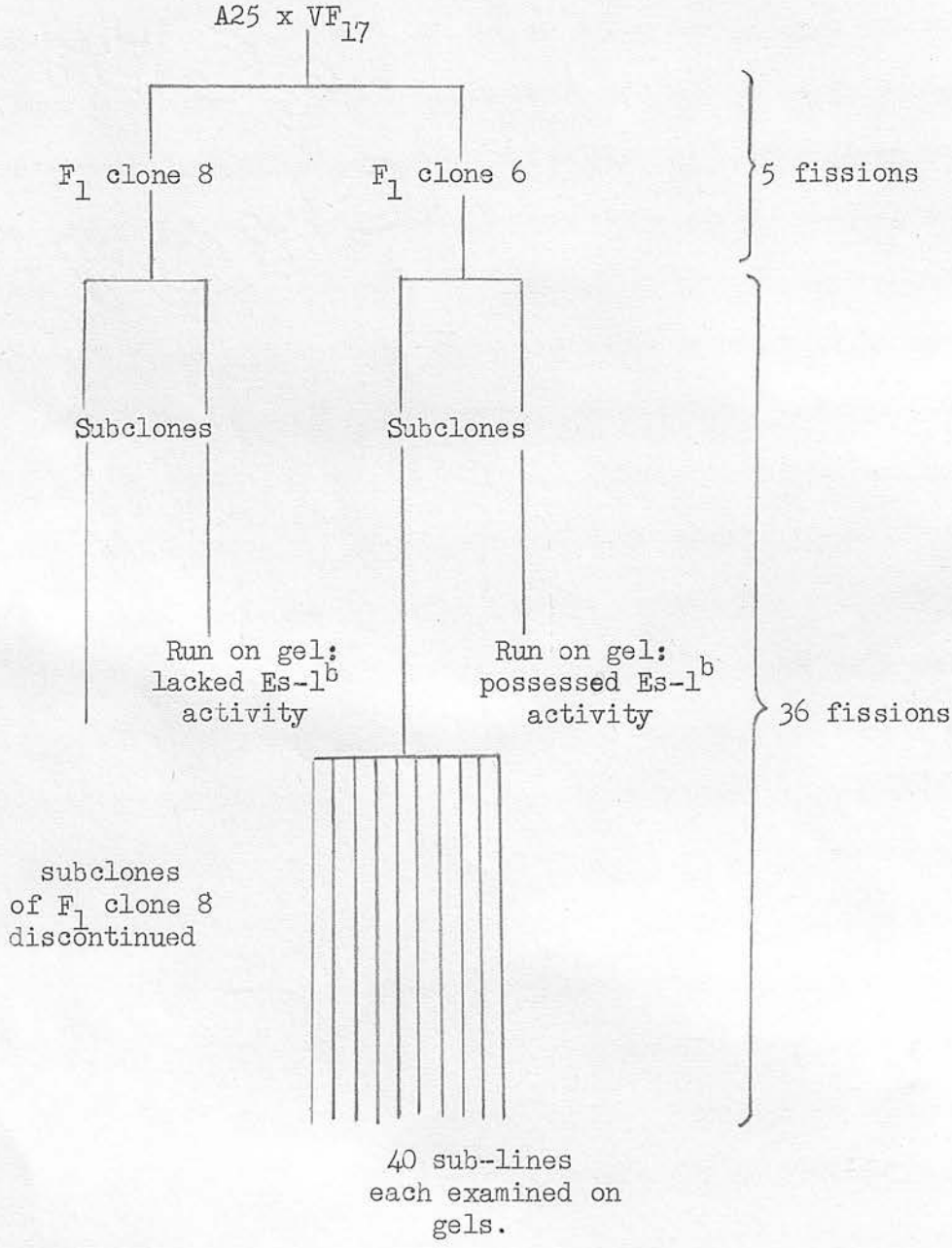
heterozygous for mating type.

During the course of the present work intraclonal conjugation was seen on two occasions. It was first observed in two F_1 clones (from the A25 x 20 cross) when they were six months old. The genetic analysis showed that these clones were heterozygous for the mating type genes. It was therefore possible that the new mating type had arisen as a result of autogamy. With a view to testing the mating types of both co-conjugants, pairs from one clone were split during the very early stages of conjugation and the pre-conjugants reisolated. In 6 cases both members of the split pair survived and divided. After one week, during which autogamy did not occur, pairs were present in 7 of the 12 cultures. From Table 14 it is seen that 4 of these selfing cultures were derived from both members of two split pairs whereas the other 3 selfing clones arose from only one member of a split pair. It is evident that conjugation can sometimes occur between animals which are isogenic. The observation that both members of a split pair can produce selfing cultures means firstly, that the change in mating type which allowed selfing in this clone was not due to a stable genetic change arising from a segregation of mating type alleles at some previous autogamy. Secondly, there is not a stable change in gene expression such as occurs in Tetrahymena and in Paramecium (see review by Preer, 1969), but it is due rather to a temporary breakdown in dominance, such that the usually recessive allele comes to be expressed.

On the second occasion intraclonal conjugation was observed in an F_1 clone of genotype mt^{11}/mt^{7} on achieving sexual maturity at the age of about 60 fissions. Autogamy had not occurred in this clone prior to this time, although it was observed almost simultaneously with the appearance of selfing. 200 autogamous animals were isolated and the 2 which survived to give F_2 lines did not exhibit the selfing trait. Nobili and Luporini (1966) found that exautogamous lines derived from a selfing clone may themselves show intraclonal conjugation.

Figure 4

Outline of experiment to investigate intraclonal variation at the Es-1 locus

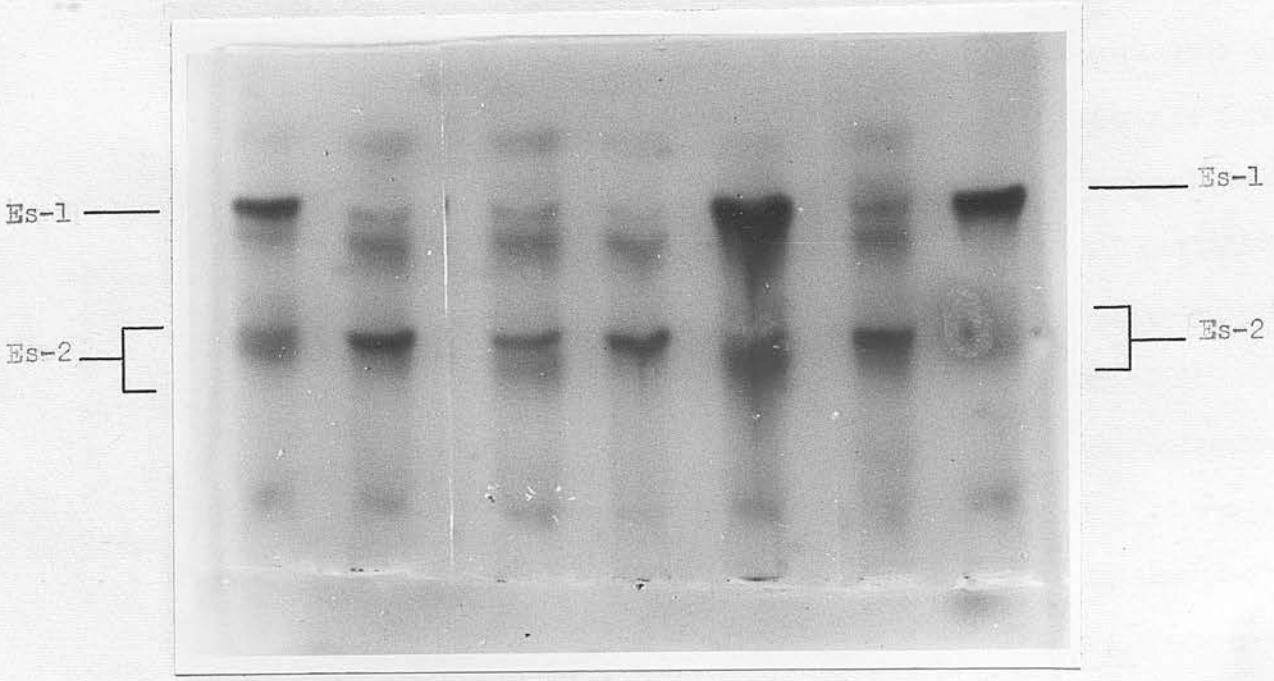


The observations above showed that transitory changes in gene expression can occur in E.minuta. However the rarity of selfing clones in comparison with the abundance of such clones in E.crassus implies that both short-lived changes, and the stable genetic changes observed for six loci in Tetrahymena (Allen and Nanney, 1958; Nanney, 1964; Allen, 1965; Phillips, 1967), do not occur for the mating type alleles at any appreciable frequency in E.minuta.

It is with changes of this more stable sort in mind that the following investigation was designed. The outline of the experiment is given in Figure 4. Several F_1 clones from the cross A25 x VF_{17} were expanded into sub-clones initiated from single cells at the age of about 5 fissions. When the F_1s were typed for esterase activity, several of these clones were found to lack Es-1^b activity. The experiment was continued with one clone (clone 6) which did possess Es-1^b activity and must be heterozygous at this locus. After the age of approximately 40 fissions, 60 new sub-lines were started from single isolates of one of the sub-clones of clone 6. 40 of these sub-lines were grown into mass cultures, harvested, and the homogenates run on starch gels. Up to the time of harvesting, autogamy had not been observed in any of the sub-lines. Efforts were made to maintain uniform culture conditions and to standardise the concentration of homogenates.

Figure 5 is a photograph of one of the gels. All 40 sub-lines of F_1 clone 6 possessed Es-1^b activity, but at enormously varying levels. The fact that not one sub-line expressed exclusively the silent allele means that stable phenotypic drift either does not occur in E.minuta for this allele, or else occurs at a detectable frequency only after 40 fissions. The variation in gene expression could be a result of:

Figure 5



Starch gel zymogram showing the esterase activity of 7 isogenic sub-lines of a clone of genotype Es-1^{a}/Es-1^{b}. The photograph illustrates the variation in gene expression at the Es-1 locus.

a) uncontrolled heterogeneity in the conditions of culture,

b) stable changes in gene expression, but with complete "switching off" of one allele occurring only after 40 fissions, or

c) transitory changes in gene expression.

Whatever the explanation for this quantitative variation in gene expression, it would be unwise to attempt to distinguish between the homozygote ($Es\text{-}1^{b}/Es\text{-}1^{b}$) and the heterozygote ($Es\text{-}1^{a}/Es\text{-}1^{b}$) on starch gels. Although efforts to correlate the staining intensity of esterase bands with homo- or heterozygosity have sometimes been successful (e.g. Petras, 1963) other attempts have failed (Pelzer, 1965; Randerson, 1965).

PART III

The Cytology of Nuclear Reorganisation

a) The process of Conjugation.

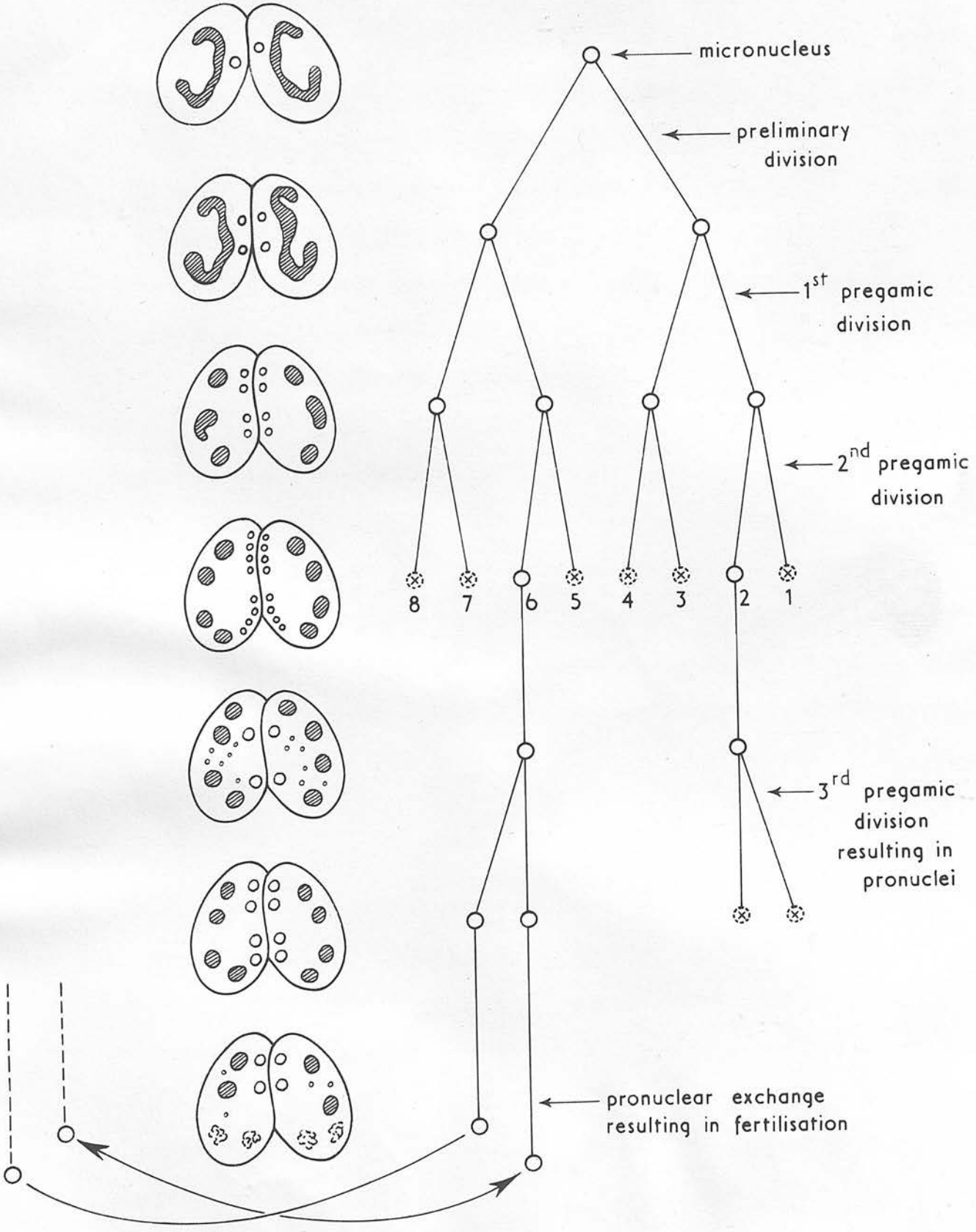
After a phase of logarithmic growth and a reduction in the food supply, stocks of different mating types conjugate. The immediate clumping observed in P.aurelia (Sonneborn, 1950) is absent, and pairs are not formed for at least 1 to 2 hours after mixing. The time required for mating seems to depend not only upon food supply, but also upon the genotypes of the stocks used. The autogamous stocks may take up to 5 hours to mate (Luporini and Nobili, 1967a). Stocks 11° and 16 were regularly used for studying the conjugation process, as firm pairs are formed after 1½ hours. Before pair formation, pairs of animals are seen swimming spirally around one another. They then settle and as the pairs become firmly united along their left ventral surfaces they become less active. As the conjugation process proceeds the posterior part of the AZM is lost and only the anterior region persists. The cirri are also lost later in the procedure. Both the AZM and the cirri are replaced by the time a new macronucleus has been reorganised (Maupas, 1889; Turner, 1930).

b) Nuclear events at Conjugation.

Figure 6 gives a summary of nuclear events during conjugation. In stocks 11° and 16 no nuclear change was observed in the pairs for about 3½ hours. The first sign of activity is the migration of the micronucleus out of its depression in the macronucleus. The interphase micronucleus is about 1.5μ in diameter. It appears homogeneous when Feulgen-stained and has a distinct boundary due to the presence of the nuclear membrane (Plate 2,A). Turner (1930) described a vesicle around the micronucleus in a species of euplotes

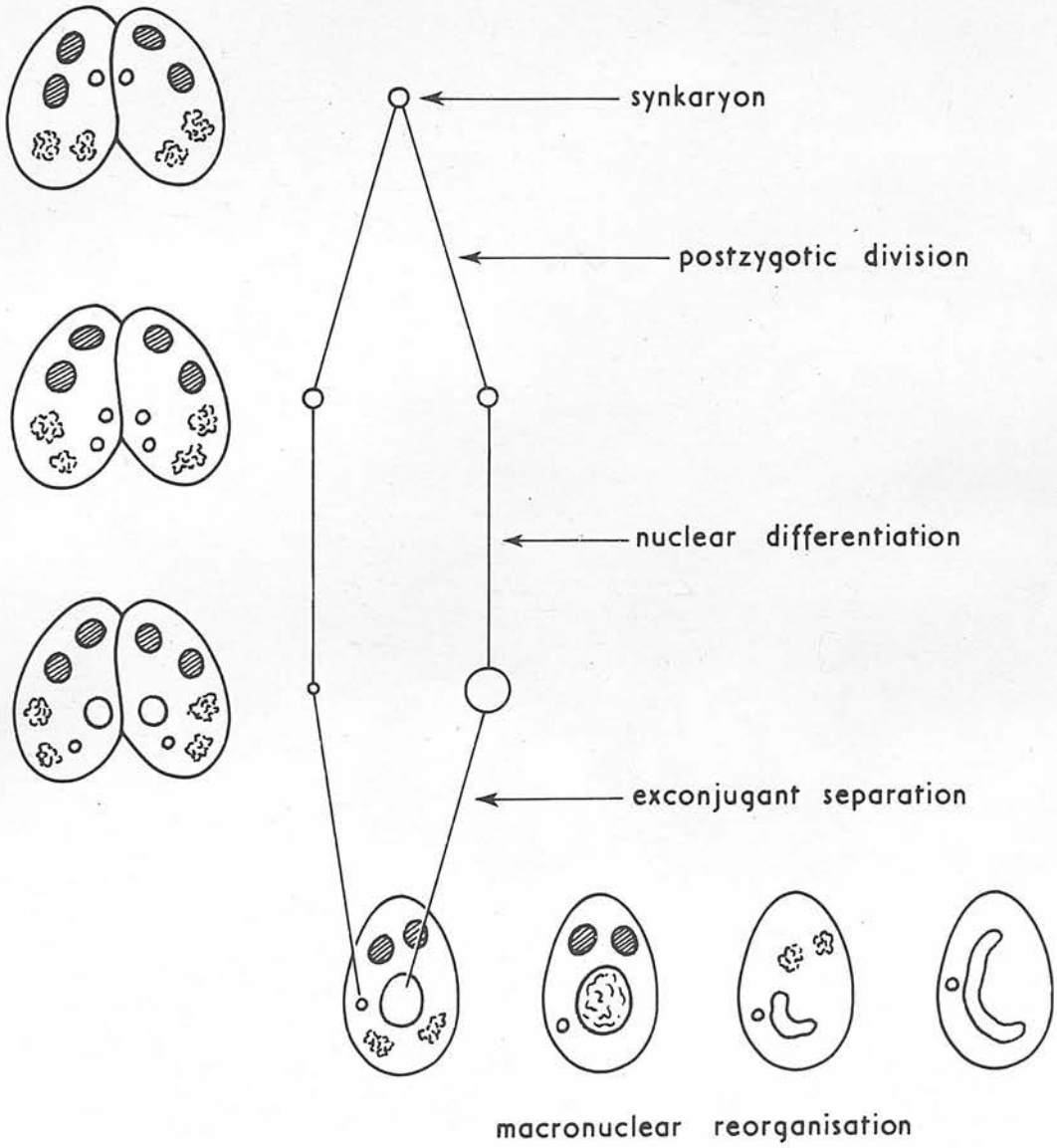
Figure 6

A summary of the nuclear changes during conjugation, and nuclear reorganisation in exconjugants in Euplotes minuta.



Incomplete circles represent degenerating micronuclear products.

Figure 6 (continued)



which he called E.patella, but which Pierson (1943) identified as E.eurystomus. Both Rao (1964) and Wichterman (1967) showed diagrams of a halo around the micronucleus in E.woodruffi and E.cristatus respectively. This was possibly an artefact due to shrinkage at fixation, and nothing similar was observed in this study. Before the preliminary division of the micronucleus in E.minuta it swells to 3μ in diameter (Plate 2,B). The first division resembles a vegetative mitosis of the micronucleus, except that there are usually no reorganisation bands traversing the macronucleus at the same time. The two resulting micronuclei enlarge and stain lightly with nuclear stains (Plate 2,C).

The macronucleus begins to fragment 5 hours after pair formation. The chromatin starts to constrict at two points (Plate 2,D) to produce an elongated anterior lobe and 2 posterior segments, all of which are at first connected by a thin strand of nuclear material (Plate 2, E and F). The anterior lobe later fragments to two portions and finally the fragments become quite separated from one another. Although usually 4 fragments result occasionally 3, 5 or 6 are seen. Fragmentation takes 1-2 hours and is nearly completed when the micronuclei divide further.

7-8 hours after pair formation the micro-nuclei are undergoing the 1st pregamic division. The micronuclei have continued to swell since the initial mitotic division and are now 5 to 6μ in diameter. This division is morphologically quite distinct from any other nuclear division in the conjugants and closely parallels meiotic divisions in other ciliates (Plate 3, A, B and C).

Spindle fibres stretch from pole to pole, orientated parallel to the longitudinal axis of each conjugant. Between 12 and 40 Feulgen positive rods or granules are visible on the equatorial plate. These are about 0.1μ in size and are therefore at the limit of resolution of the light microscope.

Occasionally they appear to aggregate so that a small number of units (4-8) are seen at this and subsequent divisions. At anaphase the chromosomes can be seen moving towards the poles which remain connected by the spindle fibres (Plate 3,D).

Estimates of chromosome number in the genus Euplotes present rather an ambiguous picture. Griffin (1910) saw 4-8 chromosomes during mitosis of the micronucleus in E.worcesteri; Ivanič (1929b) saw 6 chromosomes in E.patella and Turner described 8 in the mitotic micronucleus of E.eurystomus. During meiosis however, Turner used the term "chromomeres" to refer to the 32 granules seen at metaphase of the 1st meiotic division. 16 of these granules pass to each pole. At the following division however he described 8 rods, 4 of which are distributed to each daughter. In accordance with contemporary views, Turner interpreted his data as showing that the 2nd meiotic division was the reduction division.

As Turner himself said "Obviously, the interpretation depends upon the identification of the constituents of a chromosome". More recently Devidé and Geitler (Devidé and Geitler, 1947; Devidé, 1951) investigating Euplotes charon and other ciliates, concluded that true chromosomes are visible only during meiosis. At somatic mitosis, as well as at the post meiotic divisions, the chromosomes are partly or completely masked. Devidé found that structures previously described as chromosomes, seen during mitotic division, are not single chromosomes, but their aggregates. As a result of their contribution the 1st meiotic division is now known to be the reduction division in most ciliates. We can conclude from Turner's careful study of E.eurystomus that the diploid chromosome number of this species is 32. With the work of these authors in mind, Rao (1964) examined the chromosome behaviour of E.woodruffi during conjugation. On average he observed 16 bivalents at the first meiotic division, strongly

suggesting that this species shares the same chromosome number as E.eurystomus. In subsequent divisions of the micronucleus 4 rod-like structures replace the true chromosomes. After synkaryon formation 8 rods can be resolved in each nucleus.

At the 1st meiotic division in E.minuta many chromosomes are visible, but at subsequent divisions larger rods of a different character are observed. The fact that these always seem to be oriented in a longitudinal fashion gave rise to doubts that they represented chromosomes at all. Such an arrangement was also noted by Turner. The rods are clearest after the 1st meiotic division, when usually 4 are discernable. In the synkaryon 6-8 can be seen. If these are aggregates of chromosomes as proposed by Devidé then perhaps in this species also the diploid number is some multiple of 4.

The 4 nuclei derived from the 1st meiotic division are arranged in linear order parallel to the long axis of each conjugant (Plate 3,E). The fibres connecting daughter nuclei are very transitory but the 2 anterior nuclei appear to be the daughters of one division. They are spatially separated from the posterior 2 nuclei, which are also daughters.

The 2nd meiotic division, or 2nd pregamic division, follows without a resting stage to produce 8 distinct darkly - staining nuclei 1.5μ in diameter. This division is complete 11 hours after pair formation. The 8 products are, as before, arranged in a roughly linear array parallel to the long axis (Plate 3,F). The 4 anterior nuclei are set apart from the posterior group of 4 by a distance of 8μ. In each group, sister nuclei are usually arranged in pairs exactly as has been described for E.cristatus (Wichterman, 1967). Each group of 4 nuclei is thus the result of one meiosis.

Turner, studying E.eurystomus, and Katashima working with E.patella (1960) both concluded that in these larger fresh water euplotes the arrangement of the nuclei after the 2nd meiotic division is such that if they are numbered 1 to 8

starting at the anterior of the animal, with number 8 being the most posterior nucleus, then 1 and 5 are sisters as are 2 and 6, 3 and 7, and 4 and 8. In this case the products of a single meiosis are not together in a group. Instead nuclei 1, 3, 5 and 7 are derived from one meiosis and the rest are the outcome of the other meiosis. In E.vannus, E.crassus (Heckmann, 1963; 1964) and E.woodruffi (Rao, 1964) the spindles may also overlap so that neighbouring nuclei are not always sisters. The two schemes outlined in Figure 8 compare the arrangements of nuclei in the different species of Euplotes.

Let us return to the observations on E.minuta. Although the 8, presumably haploid, nuclei resulting from the 2nd meiotic division are initially alike, 2 of these nuclei swell to 3μ and stain only lightly (Plate 4,A). The remaining 6 diminish in size, become pyknotic, and are carried into the centre of the organism before they vanish (Plate 4,B). (The survival of 2 nuclei at this stage distinguishes E.minuta from P.aurelia. In the latter species only 1 haploid nucleus normally persists beyond this stage (see Jurand and Selman, 1969), and as a consequence the two gametes produced by the following division must be sisters.) The 2 survivor nuclei in each conjugant are situated opposite the survivor nuclei in the co-conjugant. Many pairs at this stage of conjugation have been examined and in almost every case 1 surviving nucleus comes from the anterior group of nuclei and the other from the posterior group. In E.minuta, they are therefore products of independent meiotic events. Table 15 presents the results obtained from the examination of 100 conjugants at this stage. In only one conjugant were both survivor nuclei derived from the same group of nuclei and in this case they were probably sisters. It is obvious that the 8 nuclei do not have equal chances of survival and that it is the second nucleus in each group that is most likely to persist. Katashima

Table 15

The survival of 2 out of 8 nuclei after the second meiotic division.

Positions of survivor nuclei	% of conjugants observed
1 and 5	10
1 and 6	5
1 and 7	2
2 and 5	6
2 and 6	26
2 and 7	8
2 and 8	2
3 and 5	1
3 and 6	15
3 and 7	11
3 and 8	4
4 and 5	2
4 and 6	1
4 and 7	4
4 and 8	2
5 and 6	1

Table 15 (continued)

Nucleus 1 survives in			17%	of conjugants
"	2	"	42%	" "
"	3	"	31%	" "
"	4	"	9%	" "

Total 99

Nucleus 5 survives in			20%	of conjugants
"	6	"	48%	" "
"	7	"	25%	" "
"	8	"	8%	" "

Total 101

(1960) also found that nuclei 2 and 6 were the most common survivors in E.patella, but in this species these nuclei are sisters. Heckmann (1964) stated that nuclei 2 and 7 most frequently survive in E.crassus.

The survivor nuclei divide again (Plate 4,C) to give a pair of pronuclei anteriorly, and a pair posteriorly. Following this division the spindle fibres persist rather longer than at previous divisions (Plate 4,D). The 4 pronuclei stain lightly and are about 2μ in diameter (Plate 4,E). 2 of these then swell slightly to 3.5μ while the other 2 shrink (Plate 4,F) and are carried into the centre of the animal where they stain intensely before degenerating. The 2 remaining nuclei are the gametic nuclei (Plate 5,A), and those in one mate are situated opposite the pronuclei in the other conjugant. It is obvious that in E.minuta, unlike P.aurelia, the gametic nuclei may be either non-sister or sister nuclei. Examination of conjugants at the stage where the gametic nuclei are swollen but the 4 pronuclei are still aligned, shows that in most cases one gametic nucleus is chosen from the anterior 2 nuclei, and this becomes the migratory gamete, while the other is chosen from the posterior pair and remains stationary. There is however, one factor which renders it impossible to predict the frequency with which gametic nuclei are sisters or non-sisters. If the 2 nuclei which survive after the 2nd. pregamic division are close to one another, there can be an overlap of the spindles at the 3rd. pregamic division which follows, so that sister pronuclei appear in positions 1 and 3, and 2 and 4 (Plate 4,D).

According to Katashima (1960) in E.patella each of the 4 pronuclei has an equal chance of becoming a gamete. In E.minuta there is a slightly greater probability that the gametes will be formed from the centrally placed pronuclei (see Table 16).

Let us consider the extreme case in which there is always an overlap of the

Table 16

The survival of pronuclei after the third pregamic division

Combinations of nuclei	No. of conjugants observed	% of conjugants observed
1 and 2	1	2
1 and 3	18	36
1 and 4	1	2
2 and 3	13	26
2 and 4	15	30
3 and 4	2	4

Nucleus 1 survives in 40% of conjugants

" 2 " " 58% " "

" 3 " " 66% " "

" 4 " " 36% " "

spindles, resulting in nuclei 1 and 3, and 2 and 4 being sisters. In this instance gametes will be sisters in 66% (36% + 30% in Table 16) of conjugants, and non-sisters in 34%. If the spindles never overlap, so that sisters are neighbours, the figures in Table 16 show that in only 6% of conjugants will gametes be sisters. The genetic data gives more elegant information on the relationship of the gametic nuclei (see Part IV).

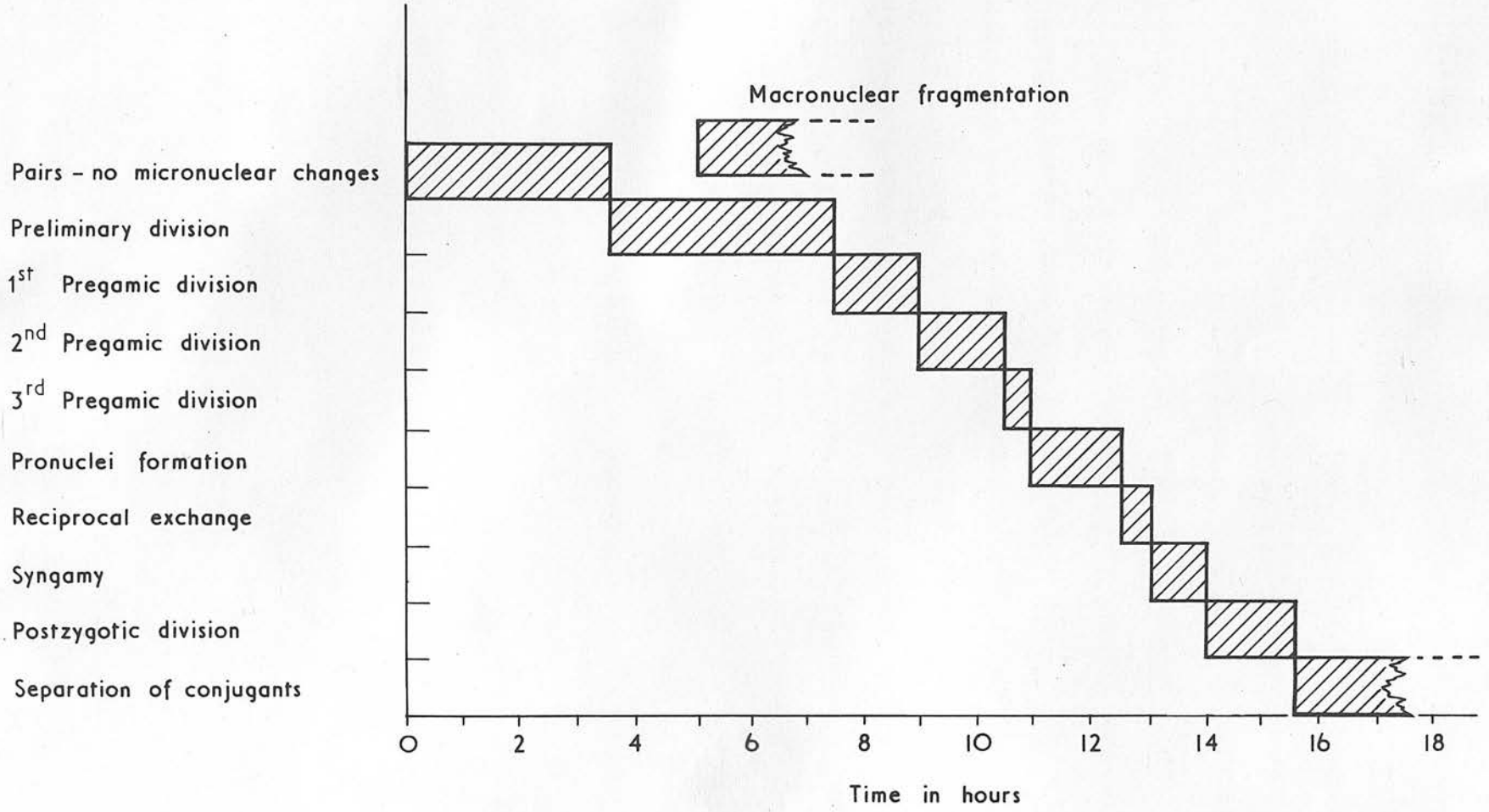
Gamete exchange occurs about 13 hours after initial pair formation. In certain cases cytogamy, or fusion of gametes within one cell, replaces cross-fertilisation. This occurs if both conjugants are out of phase in the nuclear changes preceding reciprocal exchange. Any damage to a co-conjugant which prevents the normal sequential changes, favours cytogamy. Normally the migratory nucleus passes through the cytoplasmic bridge which joins the mates for about 20μ of their length. The stationary nucleus moves up slightly from its posterior position, and synkaryon formation occurs near the mid-line, on the left margin of the cell (Plate 5,A,B,C,D and E).

There is normally one post-zygotic division which occurs 14-15 hours after pair formation, and the resulting nuclei remain attached for some time by spindle fibres which flare in the mid region (Plate 5,F). The posterior product becomes the new micronucleus, and stains intensely with Feulgen. The more anterior product, in the region of the oral primordium, becomes the macronuclear anlage. This swells rapidly to about 7μ in diameter and can only just be detected with nuclear stains (Plate 7,A). The time course of nuclear changes in the conjugants is represented diagrammatically in Figure 7.

2 post-zygotic divisions usually occur in E.patella (Katashima, 1960), E.eurystomus (Turner, 1930; Katashima, 1959b), E.harpa (Katashima, 1952), E.woodruffi (Rao, 1964), E.vannus (Heckmann, 1963) and E.crassus (Heckmann, 1964). In E.cristatus (Wichterman, 1967) as in E.minuta there is only one, although

Figure 7.

The duration of the various stages observed during conjugation in E. minuta.



Nobili (1965) stated that there are 2 post-zygotic divisions in E.minuta. There is some evidence to suggest (as is the case with E.cristatus) that a 2nd post-zygotic division may sometimes occur. Occasionally exconjugants are observed with 2 macronuclear anlagen as well as a micronucleus (Plate 6,C). Nobili (1965) has also observed this. Alternatively, in addition to the normal complement of 1 anlage and 1 micronucleus, 1, 2 or 3 extra micronuclei may be visible (Plate 6,D). These could also result from the persistence of haploid nuclei which have failed to degenerate after the 2nd and 3rd pregamic divisions, but this seems unlikely.

Because conjugation is so complex, examination of large numbers of mating pairs is bound to reveal some abnormal conjugants. After the 2nd meiotic division the maximum number of nuclei is usually 8, but in rare cases, 10, 12 and even 16 have been observed (Plate 6,A), presumably due to additional divisions of 1 or more of the haploid nuclei. Usually only 2 nuclei survive beyond this division, but occasionally 3 survivor nuclei differentiate, even in cells with the normal complement of 8 meiotic products (Plate 6, B). The aberrant conjugants may result from the centrifugation of the cultures, a process which Katashima (1960) claimed increases the number of irregularities in E.patella, but they are also likely to represent some of the naturally occurring mistakes, that inevitably arise during such an involved process.

One hour after the post-zygotic division the conjugants separate, having been attached for 15 hours (Plate 7,B). The 2 posterior fragments of the old macronucleus have begun to lose their affinity for stain and they slowly disappear. The more anterior fragments persist for some time. The exconjugants remain quiescent for at least 48 hrs. and during this time the macronuclear anlage grows in size until it occupies about 1/3 of the cell volume. Initially it is very weakly Feulgen positive (Plate 7,C), but the

affinity for stain increases, presumably co-incident with DNA synthesis.

2 days after separation the anlage becomes heterogeneous in appearance due to the presence of many Feulgen positive granules (Plate 7, D, E and F). After a further 12 hours, thick intensely staining threads can be resolved (Plate 8,A). It is difficult to say whether the threads seen in any one anlage are discrete or continuous.

Turner described a 'spireme' in E.eurystomus, but he did not appreciate its significance. In view of the many studies on the ciliate macronuclear anlage (see review by Raikov, 1969), it is credible that the Feulgen positive granules which first appear, might correspond to separate chromosomes produced by endomitotic duplication, thereby rendering the anlage polyploid. Grell (1953) has observed successive endomitotic divisions in the suctorian Ephelota, and the same process has been observed in the ciliate Spirostomum (Rao, 1968a). Alternatively polyploidy can be achieved by polyteny. The number of chromosomes may initially remain unchanged, but gradually increase in DNA content. Polytene chromosomes, with the typical transverse banding pattern observed in the salivary gland chromosomes of Drosophila and Chironomus (Painter, 1934; Beerman, 1961), would then be visible. In the ciliate Nyctotherus (Golikowa, 1965) the chromosomes also appear joined end to end, so that the anlage contains not many, but one, giant polytene chromosome. The situation seems similar in Stylonychia mytilus where only a few free ends are discernible (Ammermann, 1964, 1965). Alonso (1965) has also reported giant chromosomes in Histrio sp., Oxytricha matritensis and in Stylonychia muscorum. In the latter species the presence of about 70 giant chromosomes with transverse banding, suggests that polyteny may follow polyploidy in this species, although breakage of one chromosome during squashing cannot be excluded.

The dense spireme threads described by Turner, and the Feulgen positive threads seen in the present study in E.minuta, almost certainly correspond to the polytene chromosomes seen by Ammermann and Alonso in other hypotrichs. Recently Ammermann (unpublished results) has also observed giant chromosomes in E.woodruffi and E.eurystomus. It is possible that their presence in the macronuclear anlage is a character shared by many other ciliates in this Order. The huge DNA content of the macronuclear anlage seems to be achieved in Stylonychia and Euplotes by an initial chromosome duplication which is followed by a polytene stage when the chromosomes may become connected. However, in other ciliates (e.g. Kahlia) the process seems to occur in the reverse order (Rao, 1966).

The function of the giant chromosomes is obscure. Several attempts have been made to demonstrate RNA synthesis in the anlage (Ammermann, 1965, 1968; Pérez-Silva et al. 1969) but all have failed despite the fact that "puffing" has been observed in Stylonychia mytilus (Pérez-Silva et al., 1969), a phenomenon known to be indicative of RNA synthesis in dipteran polytene chromosomes.

At the same time as the chromosomal elements are visible in the macronuclear anlage of E.minuta, the 2 anterior fragments of the old macronucleus begin to change their appearance. They are seen to have an intensely staining centre, but the outer region is diffuse and is losing its stainability (Plate 7,F). The anlage which increases to a maximum size of 12-15μ, then shrinks and the spireme is no longer visible (Plate 8, B and C). The shrinkage of the anlage may well be a visible expression of the reduction in DNA concentration. Ammermann (1965; 1968) has measured this reduction spectrophotometrically and by scintillation counter measurements in Stylonychia mytilus, and also in E.woodruffi and E.eurystomus (unpublished results), and it in turn may be

associated with the loss of polytene chromosomes. In Stylonychia the loss of DNA is so drastic that its concentration is reduced to the level of a diploid micronucleus (Ammermann, 1965).

The anlage in E.minuta is now uniformly granular. As it begins to elongate and to become crescent-shaped (Plate 8, B,C and D) the micronucleus assumes its position on the left side of the organism. During the elongation of the anlage one tip is found to be more Feulgen positive (Plate 8,E). This tip which is the growing point moves forward around the left ventral margin of the animal (Plate 8, E and F; Plate 9, A). This is followed by a slight elongation of the other tip around the posterior margin (Plate 9, B and C). The reorganisation process appears to follow the same pattern in E.eurystomus (Katashima, 1953). The two remaining macronuclear fragments diminish in size and stainability until they have both completely disappeared, one generally vanishing before the other. Turner reported that the anlage routinely fused with an old macronuclear fragment in E.eurystomus. Katashima (1953), re-examining macronuclear reorganisation in this species, concluded that fusion does not occur, and that Turner was misled by the overlap of the growing tip of the anlage with the old fragments. Rao (1964), claimed that the developing anlage in E.woodruffi fused with remnants of the old macronucleus to form the T-shaped macronucleus, but his later autoradiographic work on this species (1968b) did not substantiate his previous result. Faure-Fremiet et al. (1954) also reported the fusion of fragments with the anlage in E.musicola, but this has not been confirmed (or disproved) by independent study.

In exconjugants of E.minuta, as in those of E.patella, E.harpa and E.cristatus, fusion of the anlage with macronuclear fragments has never been seen, neither were they seen to overlap. Certainly the evidence is against such a fusion in exconjugants of all species which have been studied genetically. The suspicion

that such a process might occur led Powers (1943) and Sonneborn (1947) to speculate that the genetic effect of the old fragments was nullified. However in the species studied so far, with the possible exception of E.musicola, the fusion of the old remnants with the new anlage seems to be more apparent than real.

During the elongation process the anlage appear to contain fibrils which run longitudinally. It is tempting to speculate that these Feulgen positive filaments represent composite chromosomes which form a basic skeleton upon which the structure of the macronucleus is built. Seshachar (1958, 1960, 1961, 1966) has shown that the macronuclei of Blepharisma intermedium and Spirostomum ambiguum contain filaments of 150Å diameter which can be liberated by despiralising agents. He has proposed a similar explanation for his results. In these ciliates however the fibrils disappear before fission. Ringertz and co-workers (1967) also described microfibrils of 100Å diameter in the macronucleus of E.eurystomus. In Euplotes there is evidence that prior to division there is a change in the organisation of the macronucleus. Kimball, Prescott and others (Gall, 1959; Prescott and Kimball, 1961; Kimball and Prescott, 1962; Stevens,1963; Ringertz and Hoskins, 1965; Prescott, 1966) have shown that DNA is synthesised only at the leading edge of the rear zone of the reorganisation band. However, DNA labelled in this region becomes dispersed throughout the macronucleus when it condenses before division (Kimball and Prescott, 1962; Ringertz and Hoskins, 1965). It may well be that changes in the chromatin before fission exist to ensure mixing of sets of genomes.

Ammermann (1965) has observed that 5 pairs of reorganisation bands travel through the elongating anlage in Stylonychia mytilus and the same phenomenon has been reported in S.muscorum (Alonso and Pérez-Silva, 1965). Using labelled precursors Rao (1968b) detected consecutive rounds of DNA synthesis

resembling replication bands in the developing anlage of E.woodruffi. Turner also observed poorly formed reorganisation bands in the anlage of E.eurystomus. Careful searching for such visible bands failed to reveal them in the growing macronuclei of E.minuta. They may, nevertheless, exist at the level of DNA synthesis.

Reorganisation of the macronucleus and oral apparatus is finally completed 4-5 days after pair formation (Plate 9,D).

c) The Process of Autogamy

The appropriate stocks and hybrids undergo autogamy after a certain interautogamous period. When the cells are competent, autogamy is induced by the same factors which produce mating in mixtures. The onset of autogamy cannot be detected whereas the onset of conjugation is marked by pair formation. Therefore the trait was examined in cultures in which both the appropriate conditions, and the presence of some animals with visible anlagen, suggested that many animals would be in the early stages of autogamy. It is impossible to assign a time sequence to the events, but there is no reason to suppose that the process proceeds either faster or slower than conjugation. The AZM and other surface structures dedifferentiate, and are formed anew during autogamy.

d) Nuclear events at Autogamy

The micronucleus behaves at autogamy in just the same way as at conjugation. This fact has been recorded by Siegel and Heckmann (1966) and Nobili and Luporini (1967). There is an initial mitotic division (Plate 10,A) which is followed by the two meiotic divisions yielding 8 haploid nuclei (Plate 10,F; Plate 11, A).

Meiosis certainly does occur during autogamy; of that there can be no doubt. The two products of the preliminary division are frequently seen in

metaphase and anaphase (Plate 10, C, D and E). These stages are quite characteristic of meiosis in both conjugating and autogamous animals. This division differs considerably, as already stated, from any other division during the maturation process in that many small chromosomes are visible.

Two of the 8 nuclei divide again to produce 4 pronuclei (Plate 11, D and E), and two of these unite to form a synkaryon. The choice of survivor nuclei after the 2nd meiotic division appears to be controlled by the same factors which influence the survival of 2 out of the 8 nuclei at conjugation. One nucleus persists in the anterior group of 4 nuclei, and one persists in the posterior group (Plate 11, B and C). The selection of gametic nuclei after the next pregamic division also appears to follow the same pattern as that observed during conjugation (Plate 12, A and B). After synkaryon formation (Plate 12, C) there is usually one post-zygotic division (Plate 12, D), but for the same reasons presented in the previous section (namely that abnormal numbers of anlagen and micronuclei are sometimes seen in the autogamous animals) a second division may sometimes occur. The more anterior product of the post-zygotic division enlarges to form a weakly staining anlage; the more posterior product becomes the new micronucleus (Plate 12, E). The old macronucleus starts to fragment after the preliminary division has occurred, at the same time as the micronuclei are enlarging prior to the 1st meiotic division (Plate 10, B). Again usually 4, but sometimes 3 or more fragments, are formed.

Following self-fertilisation the animals are quiescent until the surface structures are reorganised. For 2 days after the post-zygotic division the anlage continues to grow and increase in staining intensity (Plate 13, A and B). At this stage exautogamous animals and exconjugants are indistinguishable from one another both in nuclear structure and in behaviour. Theanlage finally becomes granular in appearance (Plate 13, C). The granules appear to

coalesce and to increase in size and stainability, the matrix becoming Feulgen negative. The granules appear to be connected into a spireme as is seen in exconjugants (Plate 13,D).

The majority of exautogamous animals however, never progress beyond this stage. Luporini and Nobili (1967a) have stated that 85% of the deaths following sexual processes occur during macronuclear reorganisation. This study confirmed that post-autogamous death is highest on the third day after the start of the process. This is the time at which the anlage begins to shrink and becomes crescent shaped in exconjugants. 25%-50% of autogamous isolates of parental stock A25 failed to produce viable exautogamous clones. The figure for F_1 hybrids between autogamous and non-autogamous stocks was 80%-100%. Approximately 85% of deaths are accounted for by the failure of the autogamous animals to reorganise the anlage into the sickle-shaped adult macronucleus. The remaining 15% failed to divide, or else underwent a few fissions before dying. These animals presumably suffer from a deficiency in the function of either the macro- or micronucleus, or the new oral apparatus.

Mass cultures, containing animals with a large, well-developed anlage, also contain animals in which the membrane bounding the anlage has broken, and the Feulgen positive contents, in the form of large "blobs" of chromatin have become scattered in the cytoplasm. At this stage, two old macronuclear fragments are still present. The number of animals with a disintegrating anlage increases on successive days. At this juncture it became important to distinguish between animals which had died, and animals in which a reorganisation process was continuing. Hence subsequent data was obtained by isolating animals in the early stages of autogamy, waiting until day 3, 4 or 5, and then reisolating only living animals, fixing and staining them.

With a few exceptions, disintegration of the anlage appears to be the rule after autogamy. The densely stained contents of the anlage (Plate 13, E) are released in the form of elongated or irregularly shaped pieces of chromatin. These "blobs" may be up to 25 in number and have no visible structure (Plate 13, F; Plate 14, A). The animals which survive do so by regenerating a new macronucleus from the fragments of the old macronucleus still present in the cell. One or two such fragments begin to change their appearance. It will be recalled that in exconjugants a change in the fragments at this stage is associated with degeneration; in the autogamous animals the change must be associated with synthesis. The fragments appear less dense (perhaps due to alteration in the hydration of the DNA, which affects its staining properties), and if they are close together they may fuse. Thus one or two fragments begin to enlarge and elongate. They have a reticular appearance, quite unlike the granular, elongating anlagen of exconjugants at this stage (Plate 14, B, C and F). Meanwhile, the contents of the anlage have quickly dispersed and disappeared. The result is that extraneous Feulgen positive material is rarely observed during the early elongation of the regenerating fragments in autogamous animals, whereas in exconjugants macronuclear fragments are nearly always present when the crescent-shaped anlage is of comparable size. When a single fragment regenerates it elongates first around the anterior end of the animal (Plate 14, D), and then around the posterior margin (Plate 14, E), much in the way that the reorganising anlage does in an exconjugant. A comparison between Plate 8, E and F, and Plate 14, C and D illustrates the difference in appearance of the growing macronuclei of exconjugants and exautogamous animals.

It has been mentioned that two fragments may regenerate. Although these

may promptly fuse, about one third of the reorganising exautogamous animals have two small "banana-shaped" macronuclei which may abut (Plate 15, A, B and C). Slightly larger macronuclei of rather bizarre shape are also frequent, suggesting fusion of the products of two regenerated fragments (Plate 15, D and E). Another kind of macronucleus observed is very large, very diffuse and faintly stained, as if it has achieved maximum size with a low DNA content. In this type of macronucleus reorganisation bands may be visible (Plate 15, F).

In hybrids between autogamous and non-autogamous stocks the entire nuclear process is essentially identical. Rather more aberrant animals are seen in the early stages. Survivor nuclei may fail to differentiate after the 2nd. pregamic division, and instead of 8 haploid nuclei, exceptions with larger numbers are more frequent. Alternatively only one nucleus may survive this division. Gametic nuclei may fail to develop, and extra fissions take place at this stage. Similar abnormalities at this and the post-zygotic stage can yield cells with more than one, or else no micronuclei (Plate 16, B) and with 1, 2 or 3 macronuclear anlagen (Plate 16, C). The fragmentation of the old macronucleus, which normally produces two fragments anterior to the anlage in the parent stock, can be messy in hybrids, so that numerous smaller fragments arise.

On days 3 and 4, many more hybrids die as a result of the disruption of the anlage and the subsequent failure of the old macronuclear fragments to regenerate. The majority of cells which do reorganise a macronucleus, do so by regeneration.

Until now no mention has been made of the fate of the new macronucleus formed after the post-zygotic division. A micronucleus is visible throughout

the enlargement of the anlage. During the disruption of the anlage it can usually be detected, although some confusion is possible if it lies over the region into which the contents of the anlage spill. While the macronuclear fragments regenerate, a diffusely staining micronucleus is seen. It is obvious that if the micronucleus is the product of the self-fertilisation process, but the macronucleus of the exautogamous animal is derived by regeneration, then the two nuclei will differ in their genetic composition. Most exautogamous animals will then be heterocaryons.

The cytological data cannot exclude that the micronucleus is replaced at some stage by a piece of the regenerating macronucleus containing at least one diploid genome. In this way both the new macro- and micronuclei would be genetically alike. As the phenotype of all the characters studied is largely, if not entirely, determined by the genes in the macronucleus, the genotype of the micronucleus can only be ascertained by crosses. Extensive crosses of exautogamous clones can determine whether the micronucleus shares the genetic constitution of the macronucleus. There is unfortunately very high mortality in such crosses. Nobili and Luporini (1967) performed 12 crosses of this nature with exautogamous F_2 animals derived from a cross of stocks A25 x 20. They concluded that autogamy results preferentially in the formation of heterozygotes. A further 16 crosses with exautogamous F_2 animals produced by crossing stocks A31 x 20, led them to the same conclusion (Luporini and Nobili, 1967b). In addition their data suggest that heterozygosity is maintained in both the macronucleus and micronucleus after autogamy.

Although the majority of autogamous isolates which survive more than 3 days have regenerating macronuclei, some animals are seen in which the anlage has dispersed, but the macronuclear fragments show no sign of growth, and retain

their intense affinity for stain. Without regeneration such cells will eventually die, but they can apparently survive in a very quiescent condition for a few days. A small number of other cells appear to be reorganising the anlage in the same way as do exconjugants (Plate 16,A). These cells are completing the autogamy process without recourse to regeneration. The percentage of cells which undergo a genuine autogamy is difficult to estimate, but is of the order of 3%-10%, a figure which is similar to the proportion of segregants after autogamy of heterozygous clones.

Siegel and Heckmann (1966) described the initial nuclear events at autogamy but made no mention of macronuclear regeneration. Nobili and Luporini (1967) admitted that it could explain the phenotypes of exautogamous clones, but they did not observe regeneration cytologically. No doubt the similarity between autogamy and conjugation during the first three days led them to believe that the processes were identical during the later stages of reorganisation of a new macronucleus. Certainly the mortality during autogamy makes it a difficult phenomenon to study.

The fact that many exconjugants and exautogamous animals die when the large spherical anlage is due to be converted into a smaller, rapidly elongating macronucleus, suggests that distinct physiological and biochemical upheaval is occurring in the anlage at this stage. There are already indications that polytene chromosomes are lost and that DNA concentrations fall. It follows that considerable reorganisation of the residual chromatin into a template form upon which the polyploid macronucleus can be built, must be occurring in exconjugants. Apparently, exautogamous animals are usually unable to do this. Why autogamy should differ in this way from conjugation remains a mystery. Since the early nuclear behaviour is so similar at conjugation and autogamy one does not expect such a dramatic difference in the later stages.

Is it possible that the inviability of the anlage after autogamy is due to homozygosity? Because of the nature of the cytological events, homozygosity for all alleles is not a necessary result of autogamy. As we shall see in Part IV, if there is random fusion of the 4 pronuclei in a heterozygote after the 3rd. pregamic division, only one third of the animals will be totally homozygous, and any recessive lethal genes should only affect this proportion of exautogamous animals. Homozygosity therefore cannot account for the breakdown of so many anlagen after autogamy. If it could, one might expect the autogamous parent stock A25 to reorganise by macronuclear regeneration, but two thirds of hybrids should develop a macronucleus from the anlage, as they do after conjugation.

One must also remember that the inability of autogamous stocks, and hybrids from them, to reorganise the anlage is not a direct consequence of their ability to undergo autogamy. The genetic evidence shows that after conjugation animals with the genotype for autogamy, are quite able to develop a functional macronucleus from the anlage.

Plate 2

The preliminary division of the micronucleus and macronuclear fragmentation in Feulgen stained conjugants.

Magnification x 1050

A Pair formation has occurred. There is no change in the micronucleus which is about 1.5μ in diameter and stains uniformly.

B The micronucleus has swollen to 3μ. A small number of rods (6-8) are visible in it.

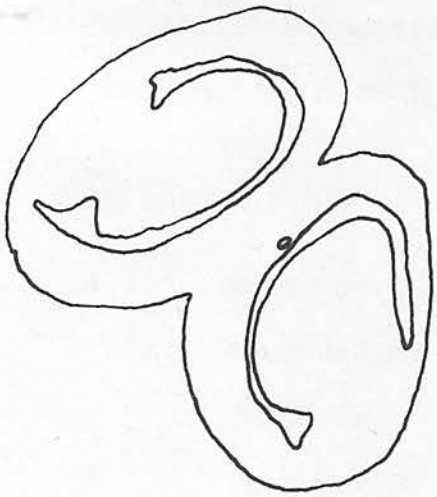
C The preliminary division has occurred to produce 2 micronuclei, one anteriorly and the other posteriorly.

D The macronucleus begins to constrict at 2 points.

E Further constriction of the macronucleus into 3 portions has occurred. These are still connected by nuclear material. The 2 enlarging micronuclei are just visible in the conjugant on the right.

F Fragmentation of the macronucleus into an elongated anterior lobe and 2 smaller posterior fragments.

A



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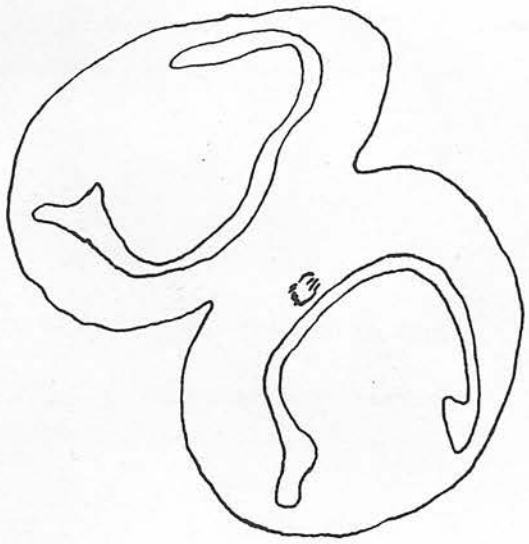

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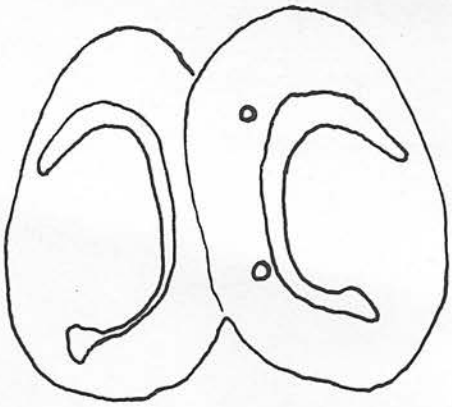

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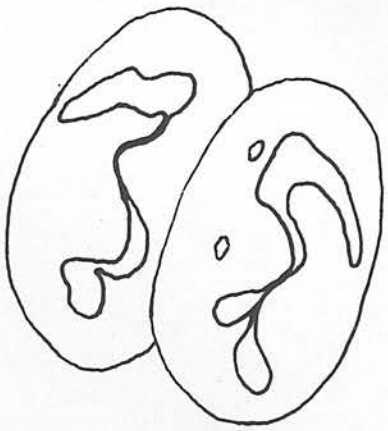

F

Plate 2

Plate 3

The 1st and 2nd meiotic divisions in Feulgen stained conjugants.

Magnification x 1050

A 1st meiotic division during conjugation. In the conjugant on the left the chromosomes are assembling on the metaphase plates of the 2 nuclei undergoing meiosis. 5 macronuclear fragments are visible.

B 1st meiotic division. In the conjugant on the left many small chromosomes are visible on the metaphase plates. Spindle fibres stretch from pole to pole. 2-3 macronuclear fragments are present.

C 1st meiotic division. In right conjugant the 2 nuclei undergoing meiosis are visible. The metaphase plates are oriented perpendicular to the long axis of the animal. 3-4 macronuclear fragments are visible.

D Anaphase of the 1st meiotic division. The chromosomes move towards the poles. Spindle fibres are oriented parallel to the long axis of the conjugant. 4 macronuclear fragments are present.

E The 1st meiotic division has produced 4 nuclei, 2 anteriorly and 2 posteriorly, each pair are thought to be sisters. 4 rods are present in each nucleus, but are not visible in the photograph.

F The 2nd meiotic division has produced a roughly linear array of 8 nuclei, a group of 4 anteriorly and a group of 4 posteriorly. Each group is thought to be the product of a single meiosis.

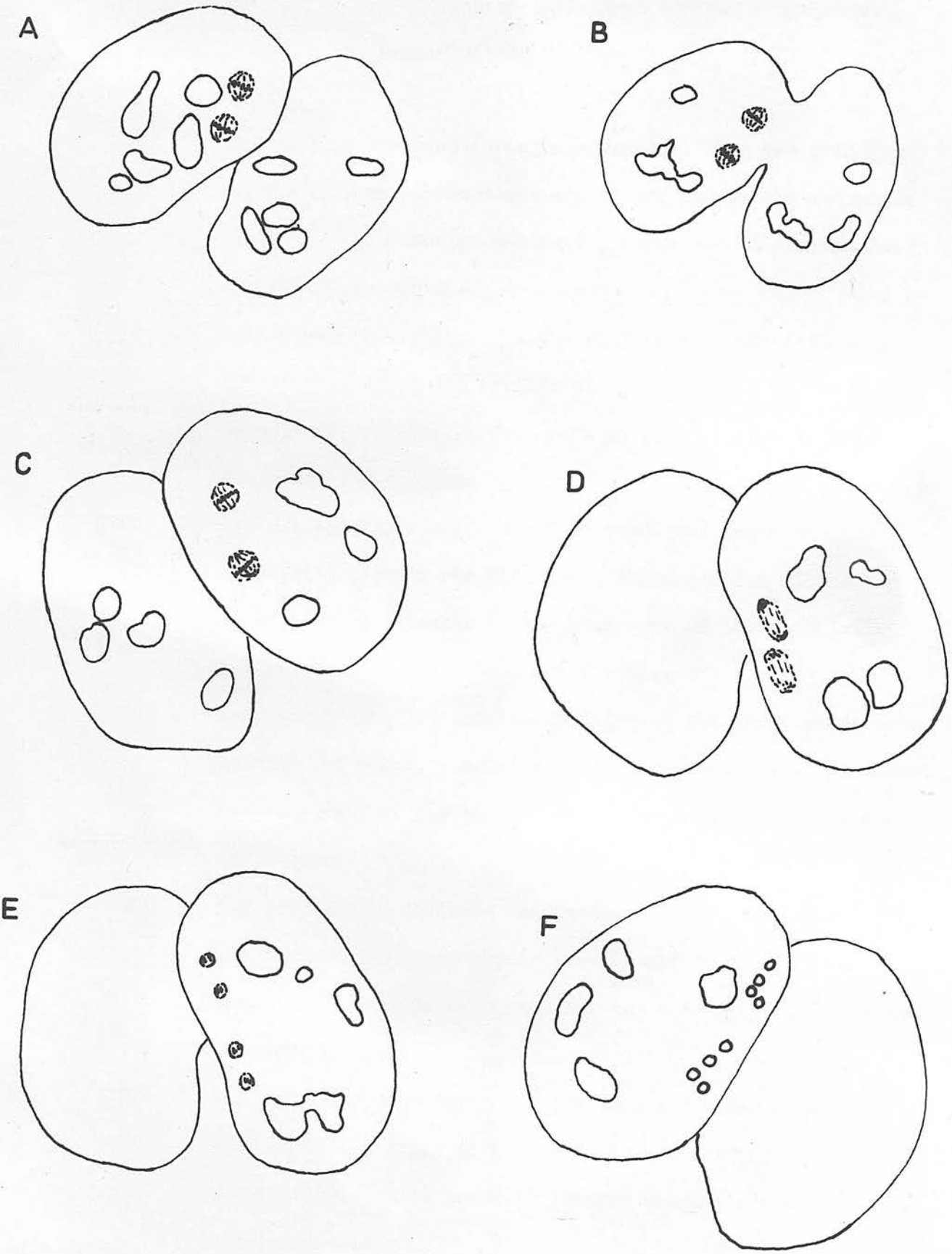
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Plate 3

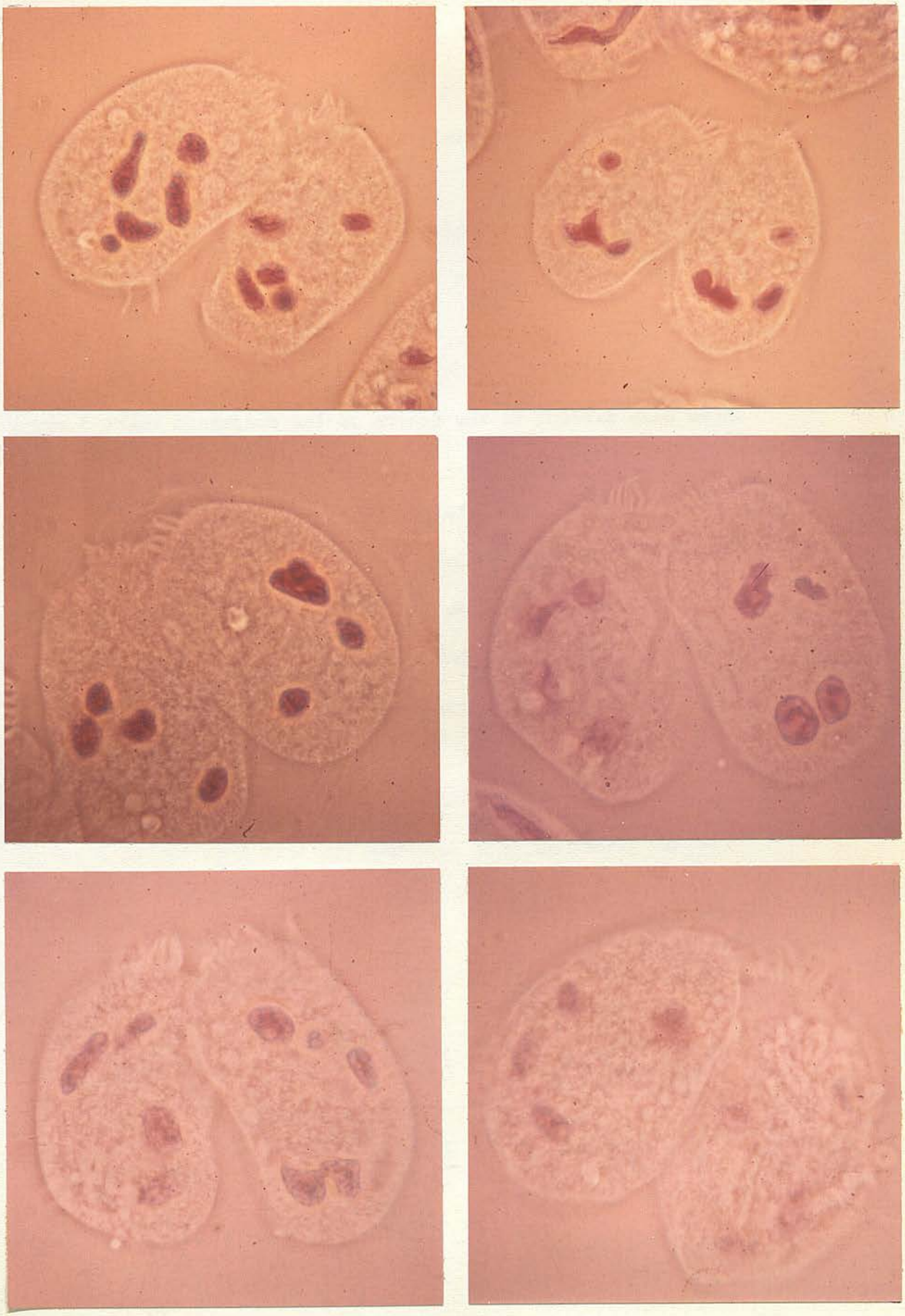

Plate 4

The survival of nuclei after the 2nd meiotic division, the 3rd pregamic division and the differentiation of gametes in Feulgen stained conjugants.

Magnification x 1050

A 2 of the 8 nuclei (in this case nuclei 2 and 6) have swollen to 3 μ and these survive to divide again. The remaining 6 nuclei stain intensely.

B The survivor nuclei are clearly visible, and the other nuclei have been carried to the centre of the conjugant where 3 are still seen.

C The survivor nuclei begin the 3rd pregamic division. The 6 pyknotic degenerating nuclei are still visible.

D The 3rd pregamic division has yielded 2 pronuclei anteriorly and 2 posteriorly. The spindle fibres persist for some time. In this case spindles are overlapping so that sisters are not neighbours.

E The 4 pronuclei are initially alike and are 2 μ in diameter.

F 2 of the pronuclei have enlarged (in this case nuclei 2 and 4), these will persist and become gametes. The other 2 nuclei stain intensely.

(The colour variation in the photographs is due to the difference in colour sensitivity of certain batches of Ektachrome colour film.)

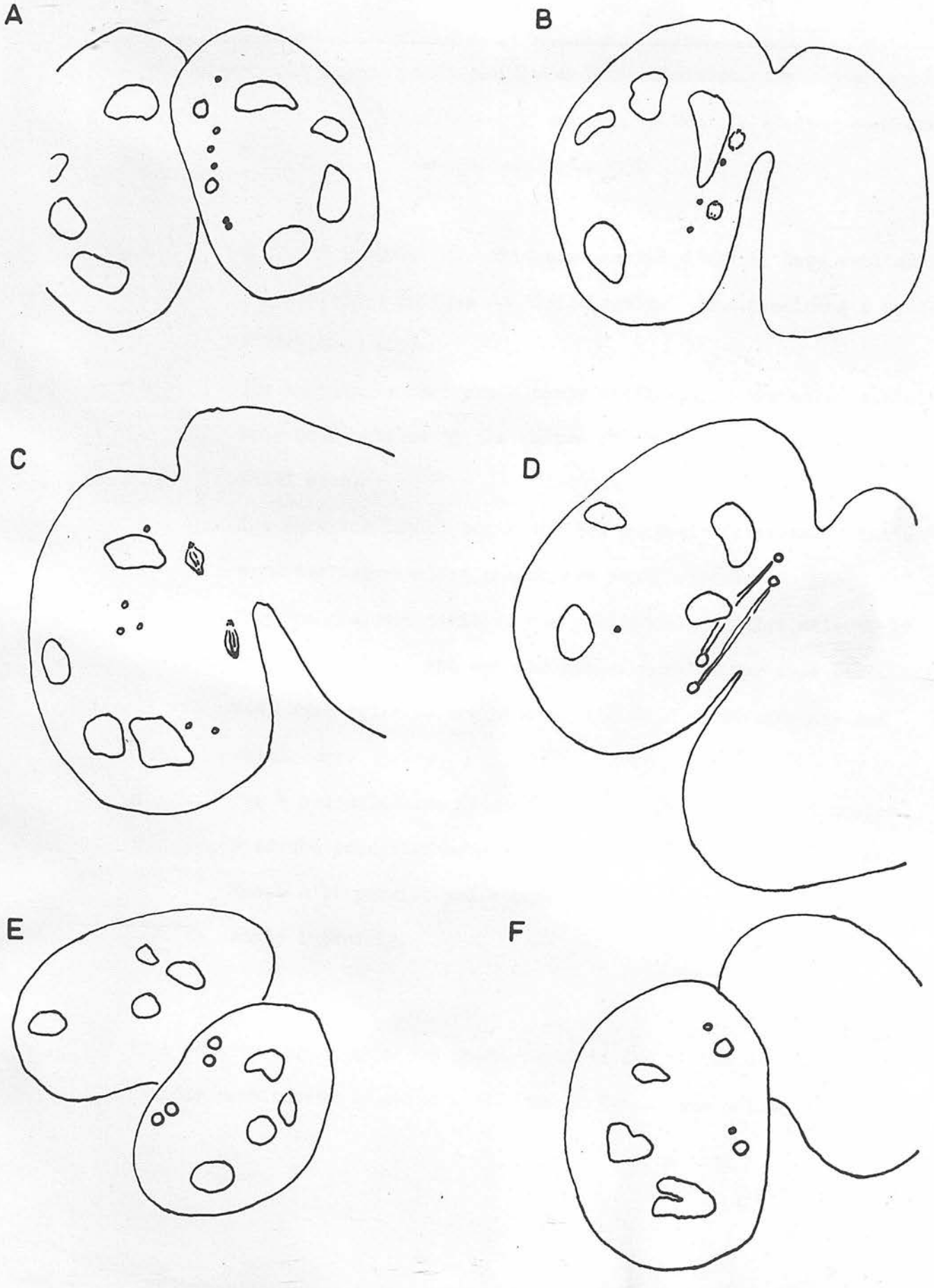
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Plate 4

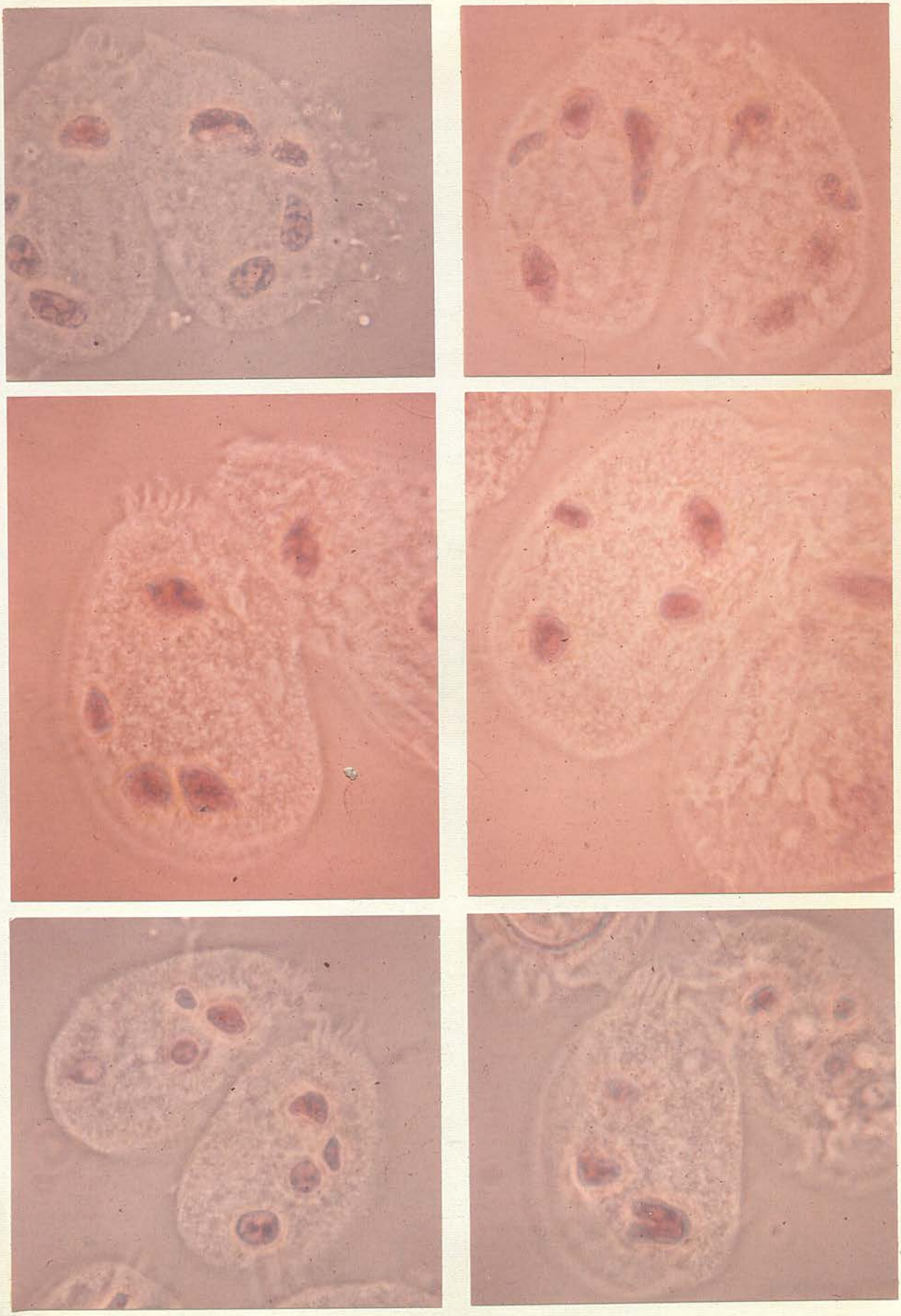

Plate 5

Fusion of gametes to form a fertilisation nucleus or synkaryon, and the post-zygotic division in Feulgen stained conjugants.

Magnification x 1050

A Two gametic nuclei 3.5 μ in diameter are visible in conjugant on the right. The anterior one is the migratory gamete, the more posterior the stationary gamete.

B Gamete exchange has occurred and fusion of gametes is about to take place in the conjugant on the left.

C Fusion of gametes occurs in the mid-region about 13-14 hours after pair formation.

D The fertilisation nucleus or synkaryon is formed. 6-8 rods are visible within it.

E The synkaryon is visible, together with 2 degenerating pronuclei from the 3rd pregamic division.

F The post-zygotic division has produced 2 nuclei still connected by fibres which flare in the centre.

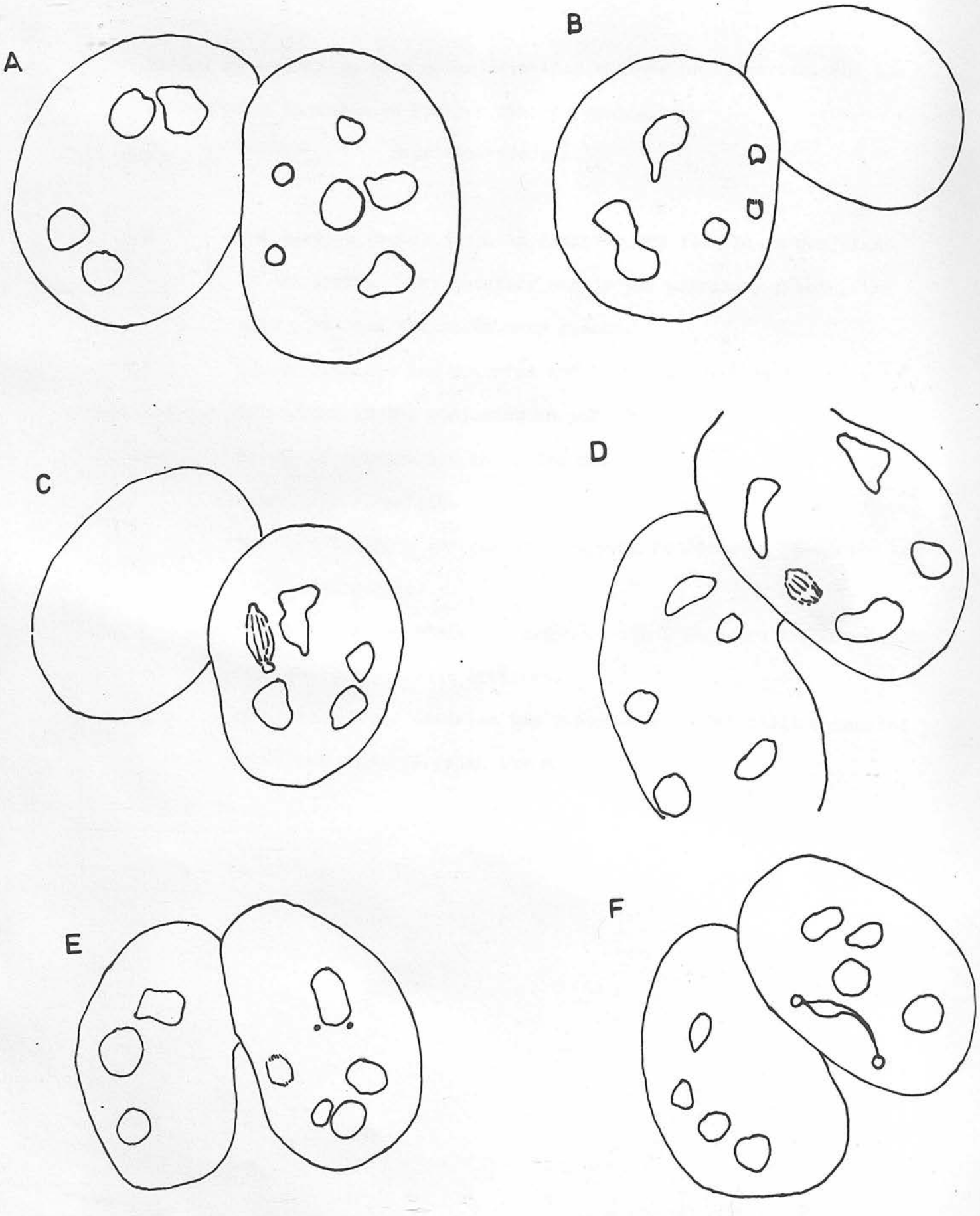
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Plate 5

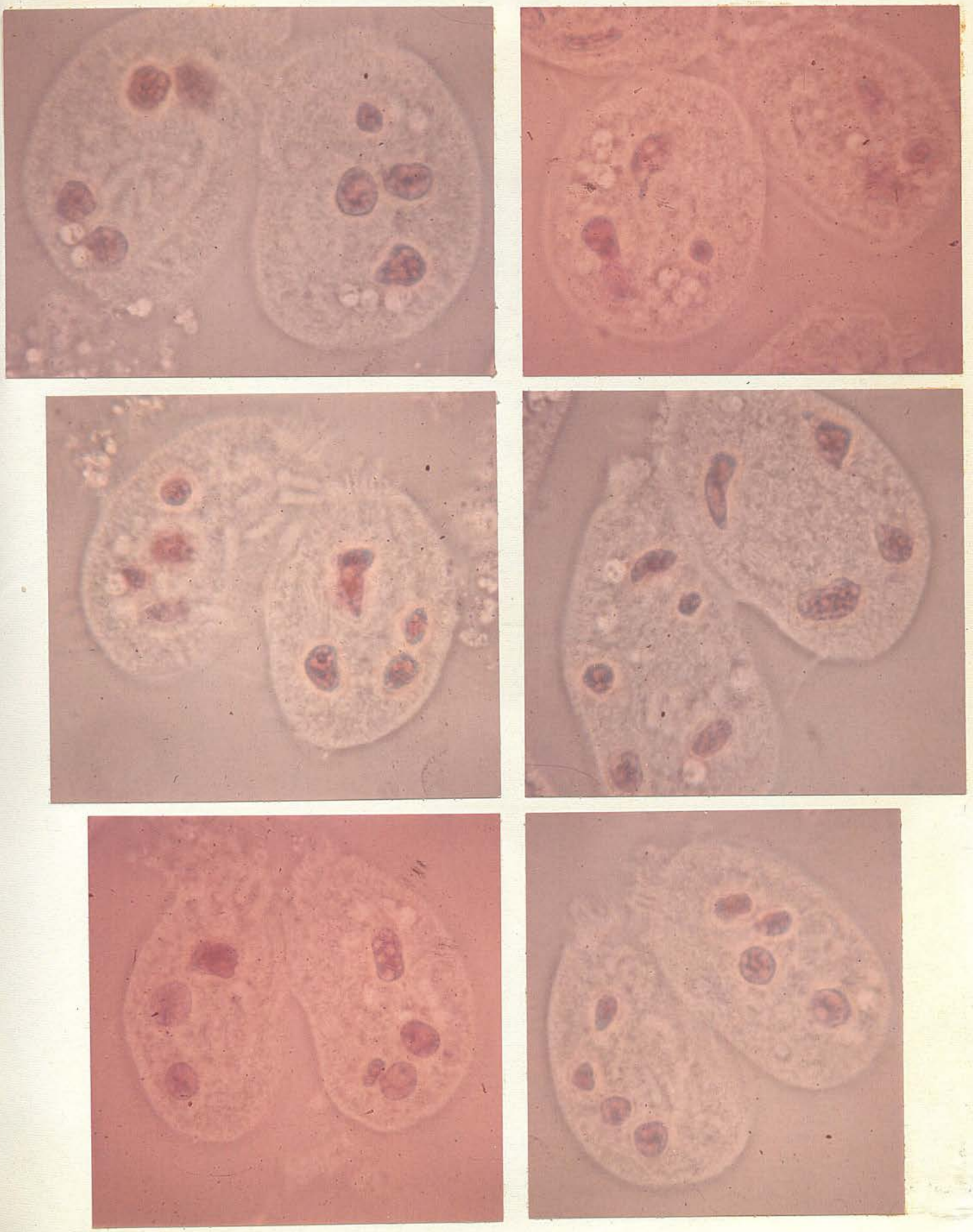

Plate 6

Abnormalities observed during conjugation

Magnification x 1050

A 12 small nuclei are visible in left conjugant, presumably due to extra division of the haploid nuclei after the 2nd meiotic division.

B 3 nuclei have differentiated after the 2nd meiotic division and are preparing to divide.

C An exconjugant with 2 anlagen and one micronucleus. This may arise if there is an extra post-zygotic division.

D An exconjugant with one anlage and 2 micronuclei can appear from the same cause.

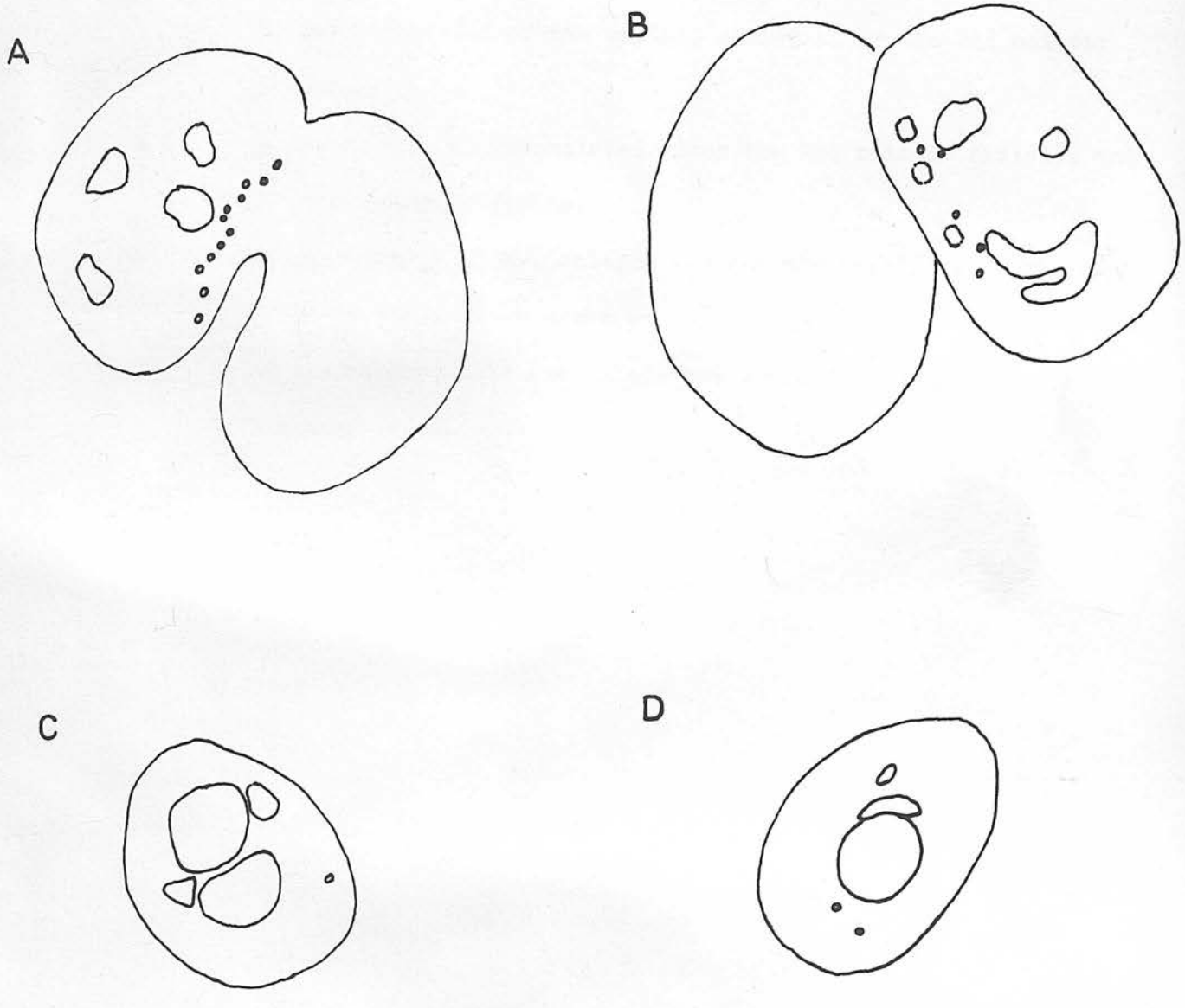
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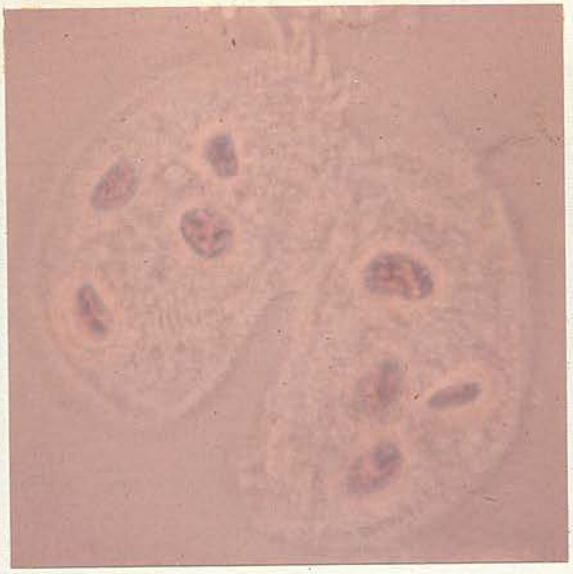
Plate 6



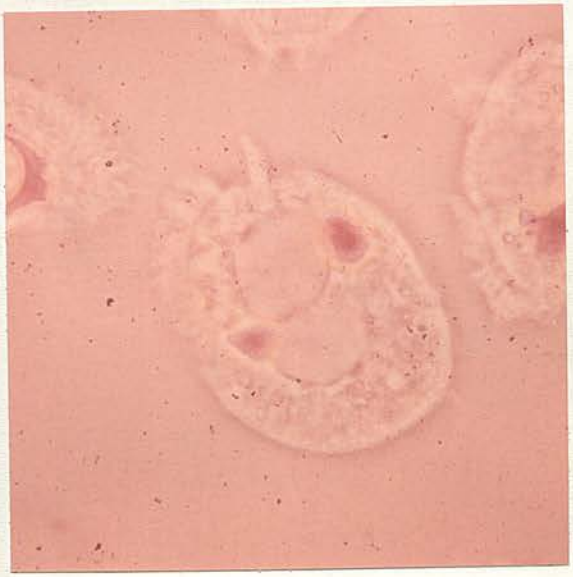


Plate 7

The differentiation of the products of the post-zygotic division. Conjugant separation and the growth of the macronuclear anlage in Feulgen stained conjugants.

Magnification x 1050

A The products of the post-zygotic division have differentiated to produce a pale macronuclear anlage and a more posterior micronucleus. The most posterior fragment of the old macronucleus is beginning to loose its affinity for stain.

B Separate exconjugant seen 15 hours after pair formation. The anlage is 7 μ in diameter. One old macronuclear fragment is dispersing.

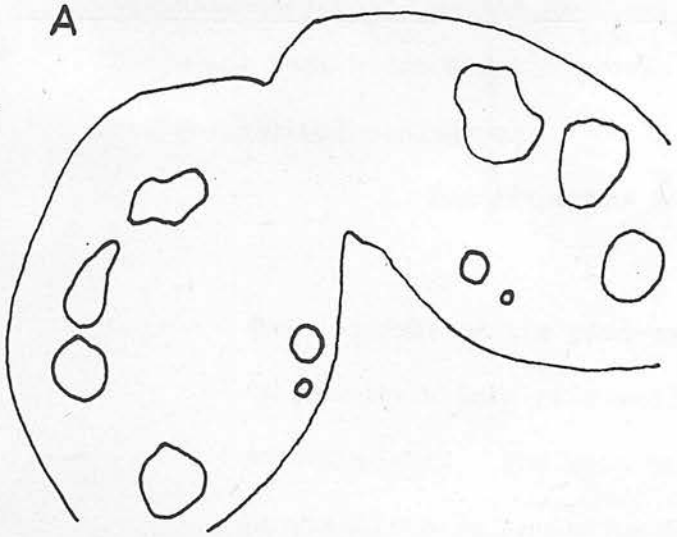
C Exconjugant about 36 hours after separation. The macronuclear anlage stains lightly and is homogeneous. It occupies 1/4 the cell volume. A single micronucleus is visible. Only 2 old macronuclear fragments persist anteriorly.

D Exconjugant 48 hours after separation. The anlage contains many small Feulgen positive granules.

E Exconjugant 48 hours after separation. The granules in the anlage have increased in staining intensity.

F Exconjugant 48 hours after separation. The many Feulgen positive granules begin to coalesce. A network of threads is just visible.

A



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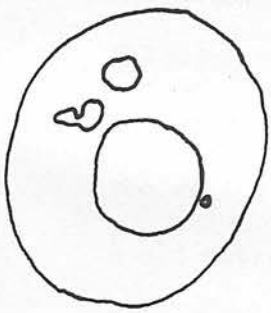

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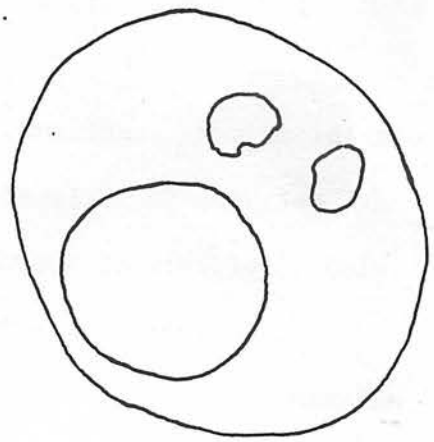

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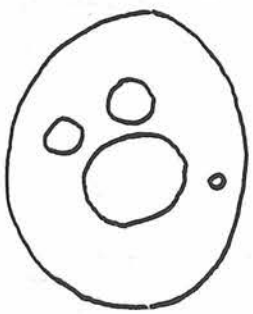


Plate 7

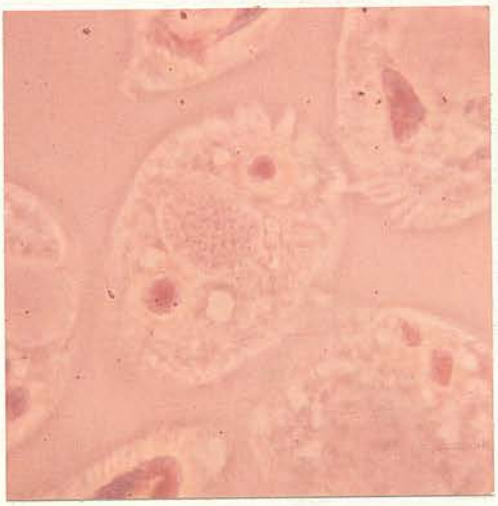


Plate 8

Reorganisation of the macronuclear anlage of exconjugants into a growing macronucleus.

Magnification x 1050

A Exconjugant 60 hours after separation. Thick intensely stained threads probably synonymous with giant chromosomes are visible in the macronuclear anlage. 2 old macronuclear fragments are still present.

B Exconjugant 72 hours after separation. The anlage has shrunk, is changing shape and stains more uniformly. A distinct spireme is no longer visible.

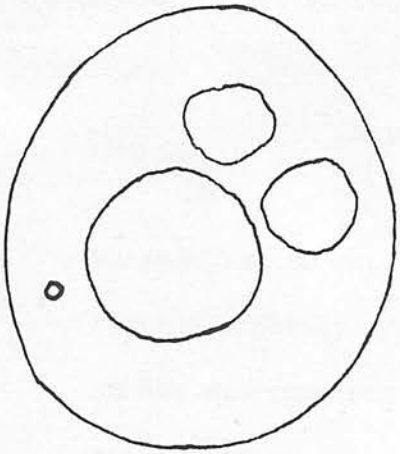
C Exconjugant 72 hours after separation. The anlage is elongating and has lost much of its stainability. The old macronuclear fragments stain diffusely.

D Exconjugant 72 hours after separation. The anlage elongates further. The micronucleus assumes its position on the left side of the organism. A single old macronuclear fragment remains.

E Exconjugant 72 hours after separation. The reorganising anlage is crescent shaped. The anterior tip, which is moving around the left ventral margin of the animal, stains more intensely. A fragment of the old macronucleus still remains.

F Exconjugant 84 hours after separation. The staining intensity has increased enormously. The anterior tip continues to elongate around the anterior end of the animal. The old macronuclear fragments have now vanished.

A



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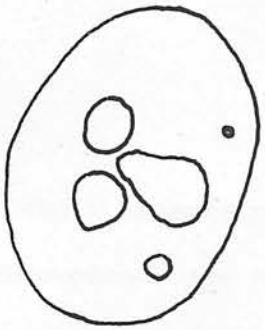

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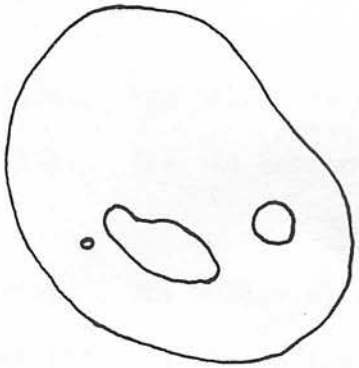

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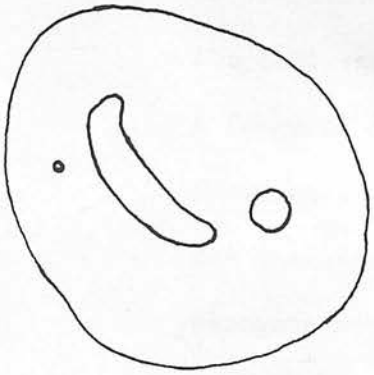

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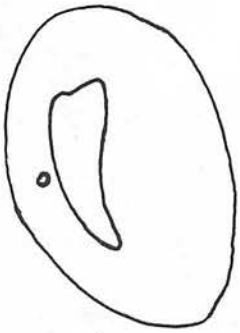
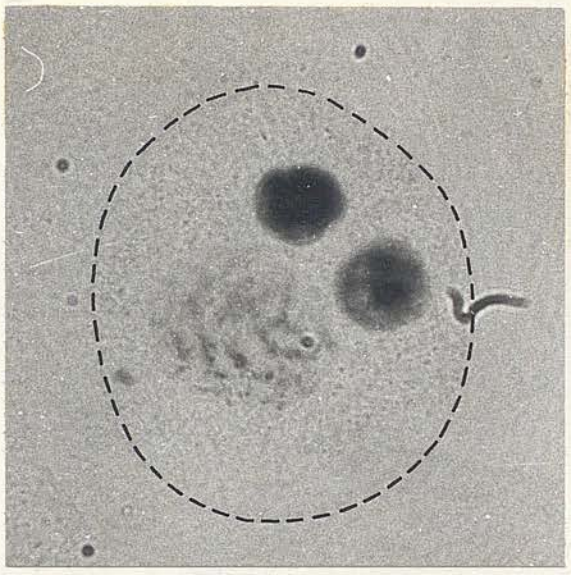
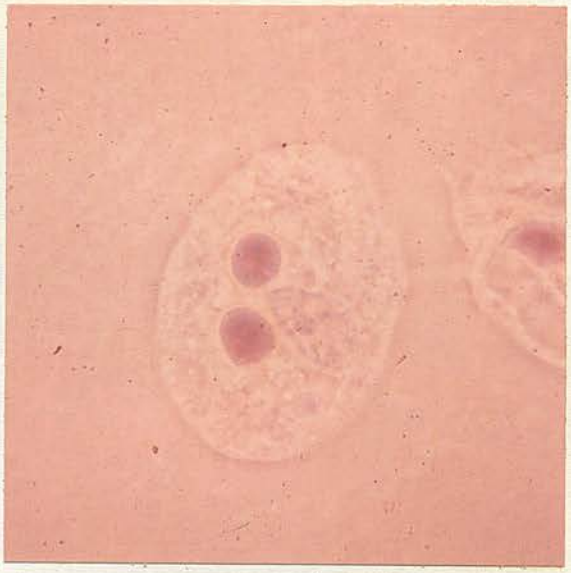
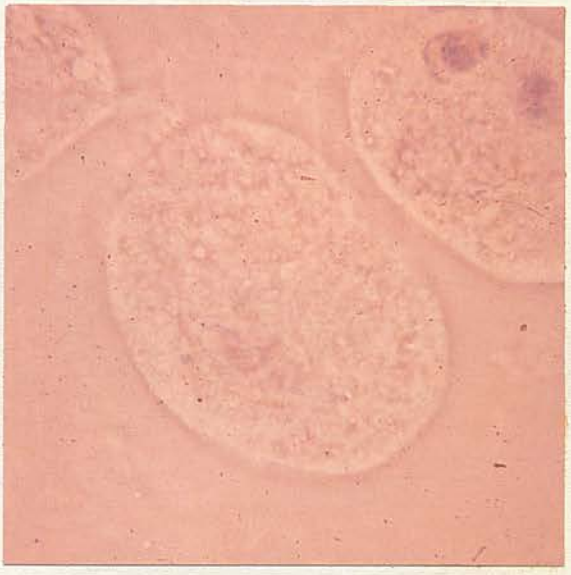
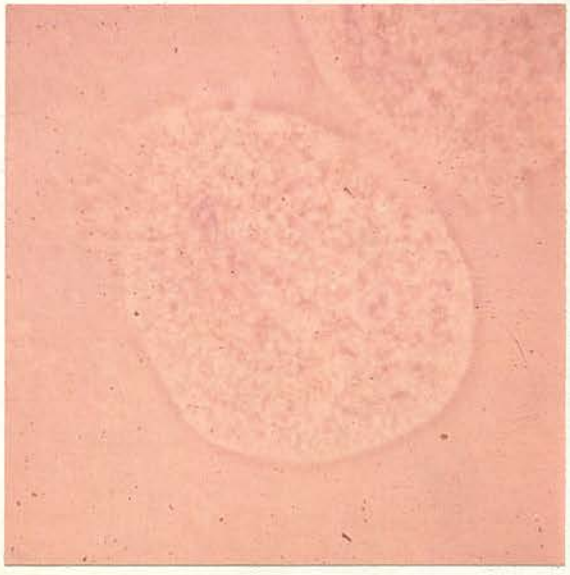


Plate 8









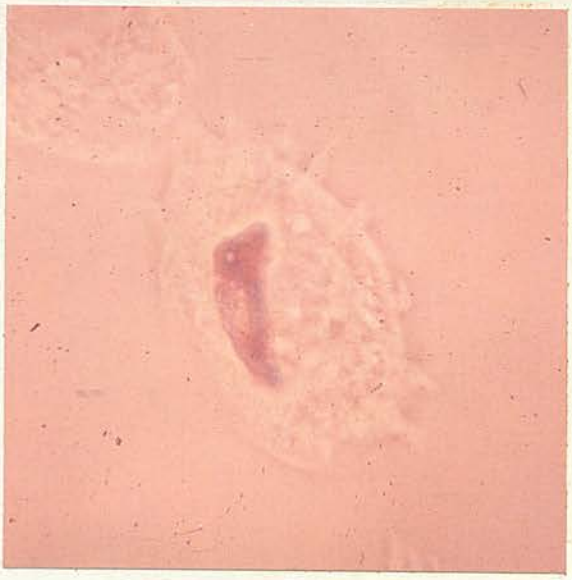


Plate 9

Final reorganisation of the macronucleus in Feulgen stained exconjugants.

Magnification x 1050

A Exconjugant 84 hours after separation. The growing macronucleus continues to elongate around the anterior of the exconjugant.

B Exconjugant 84 hours after separation. The reorganising macronucleus is now growing around the posterior margin of the animal.

C Exconjugant 84 hours after separation. Growth of the posterior tip of the macronucleus continues.

D Exconjugant 84 hours after separation. Reorganisation of the macronucleus is now complete, nearly 5 days have elapsed since initial pair formation.

A

B

C

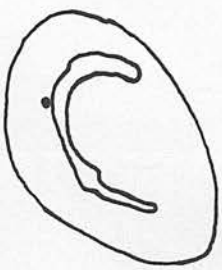


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Plate 9

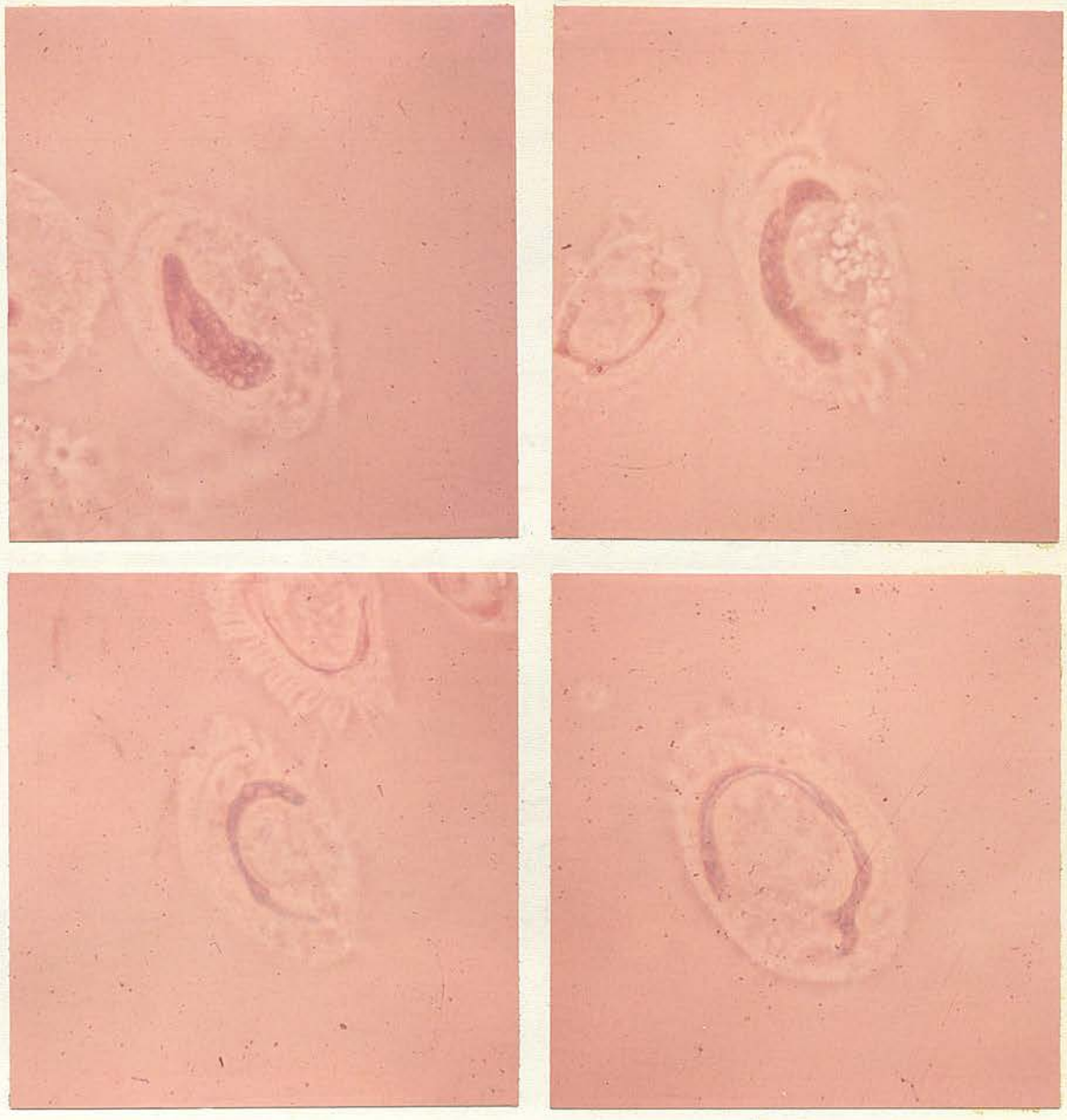

Plate 10

The preliminary division, and 1st meiotic division during autogamy. Animals are Feulgen stained.

Magnification x 1050

A Autogamous animal in which preliminary division has occurred to produce 2 micronuclei.

B The macronucleus constricts at 2 points to produce an anterior segment and 2 posterior fragments initially connected by nuclear material.

C The 1st meiotic division. Many small chromosomes are visible on the metaphase plate.

D The 1st meiotic division. Chromosomes are visible on the equatorial plate and spindle fibres stretch from pole to pole. 3 macronuclear fragments have been formed.

E Anaphase of the 1st meiotic division in an autogamous animal. Spindle fibres are oriented longitudinally. 3 macronuclear fragments are present.

F The 1st meiotic division has produced 4 nuclei arranged parallel to the long axis of the animal. The 2 most anterior nuclei are thought to be sisters, as are the 2 more posterior nuclei. 4 macronuclear fragments are visible.

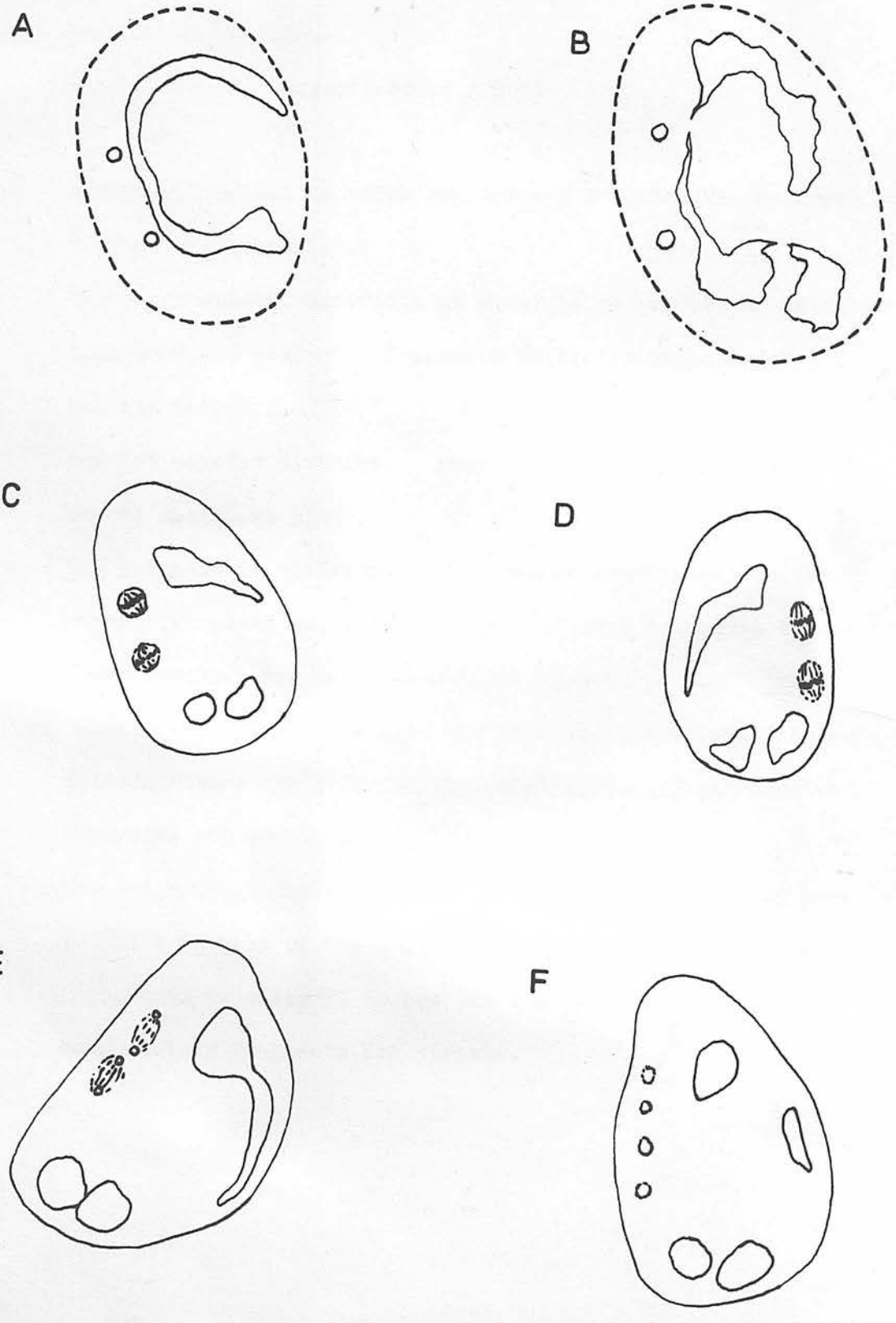
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Plate 10

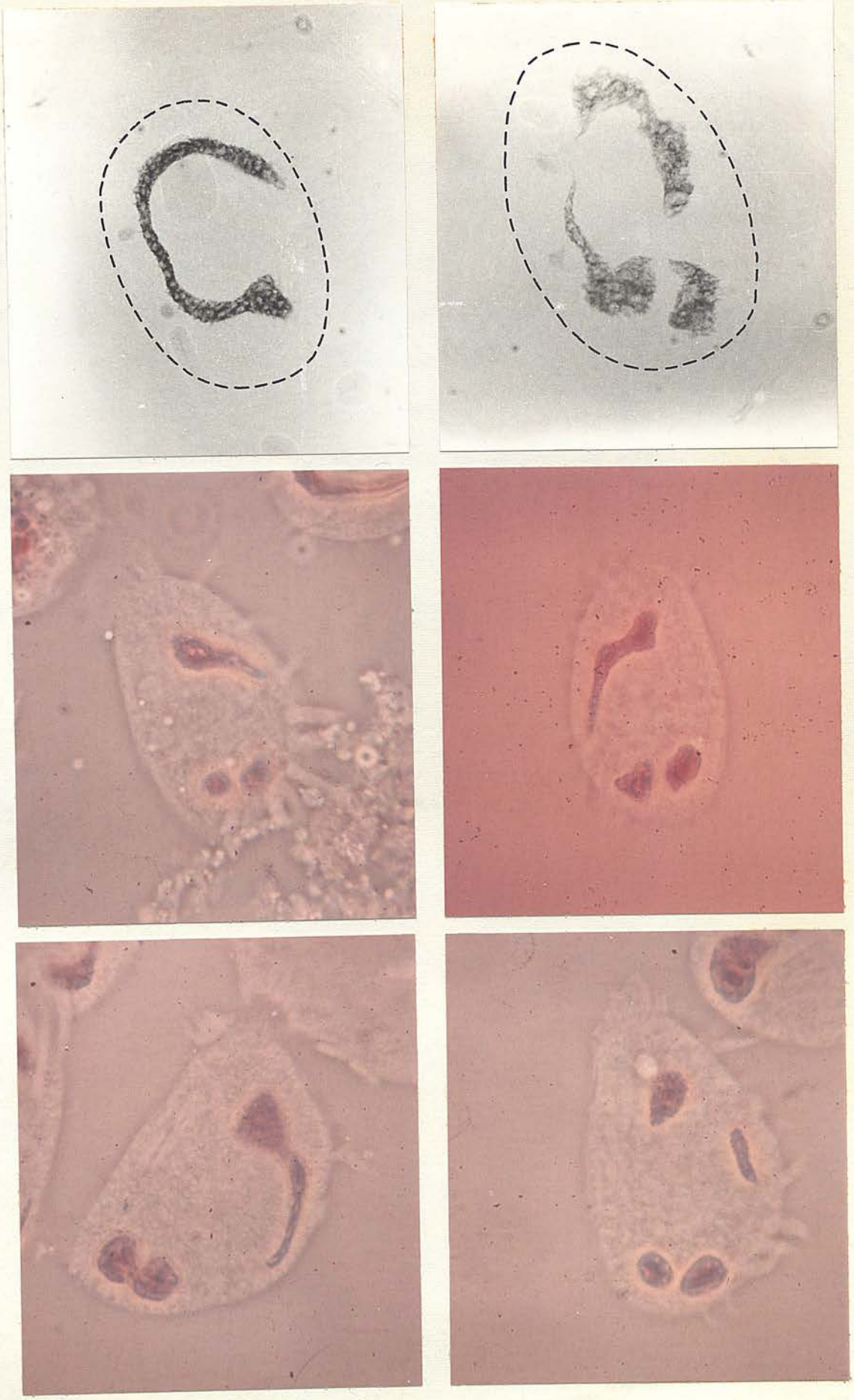

Plate 11

The 2nd meiotic division, persistence of 2 out of 8 nuclei, and the 3rd pregamic division in Feulgen stained autogamous animals.

Magnification x 1050

A The 2nd meiotic division has occurred and 8 nuclei are present. Each group of 4 is thought to be the product of one meiosis.

B 2 of the 8 nuclei (in this case nuclei 3 and 6) have swollen to 3μ. The others have begun to shrink and 2 have been carried into the centre of the animal.

C The 2 survivor nuclei are clearly visible. 5 darkly stained nuclei can be seen in the interior of the animal.

D The survivor nuclei begin to divide. This is the 3rd pregamic division.

E The survivor nuclei divide, sisters are seen connected by the spindle fibres.

F 4 pronuclei are present in this autogamous animal, 2 anteriorly and 2 posteriorly.

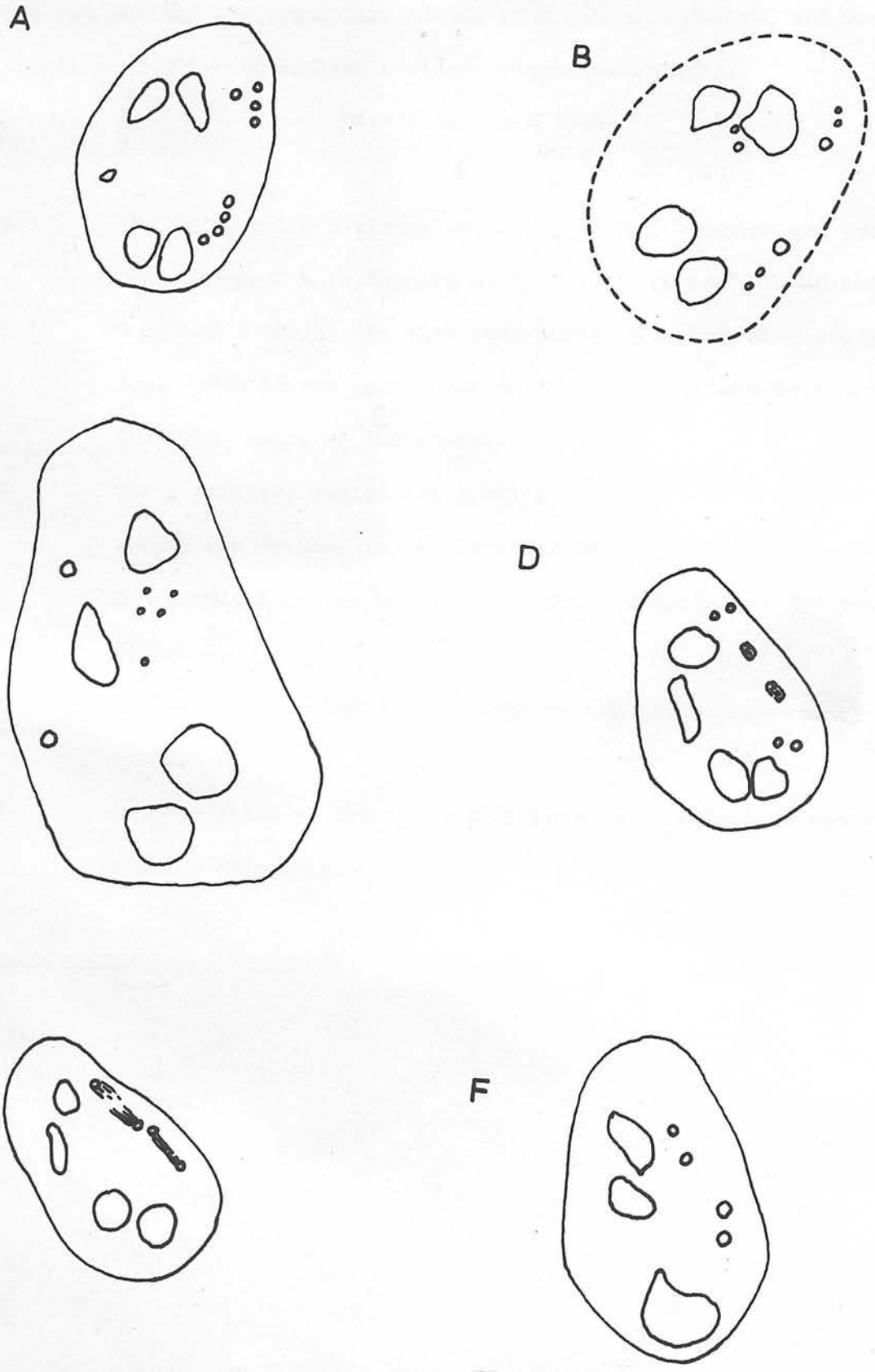
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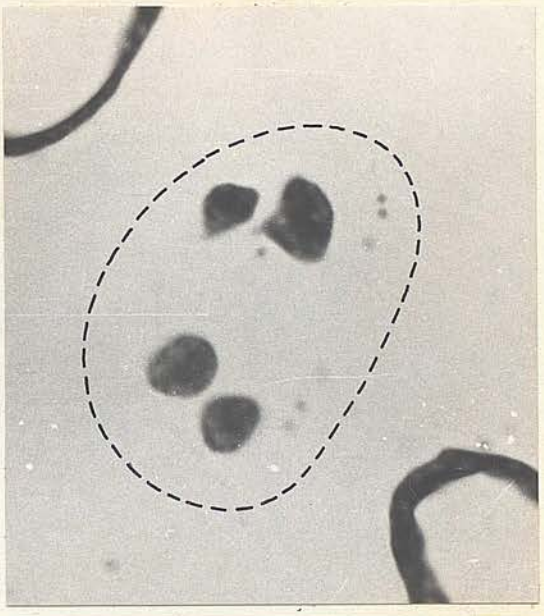
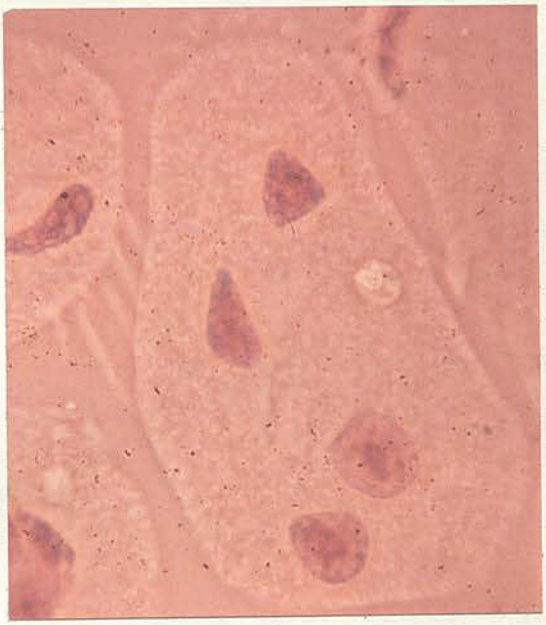
Plate 11





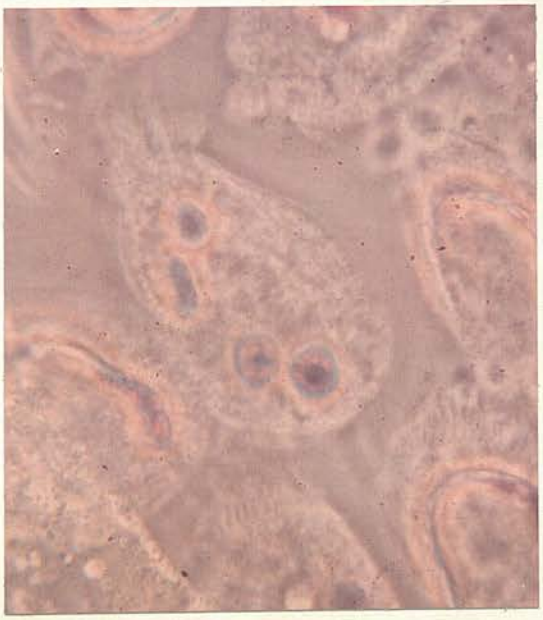


Plate 12

Differentiation of gametes, synkaryon formation and the post-zygotic division in Feulgen stained autogamous animals.

Magnification x 1050

A 2 of the 4 pronuclei have differentiated into gametes (in this case probably 2 and 4) these stain lightly and have swollen to 3.5 μ, the remaining 2 stain heavily.

B One gametic nucleus is situated anteriorly and the other posteriorly. The other 2 products of the 3rd pregamic division have vanished.

C Synkaryon formation. The fertilisation nucleus is situated near the mid-line on the left ventral margin. 6-8 rods are visible in the synkaryon.

D The post-zygotic division has occurred. The 2 products are connected by fibres which flare in the mid-region. 2 degenerating pronuclei are visible.

E The 2 products of the post-zygotic division have differentiated, the anterior nucleus has swollen, it stains lightly and is the new macronuclear anlage. The posterior product is the new micronucleus. The 2 posterior fragments of the old macronucleus are beginning to disperse.

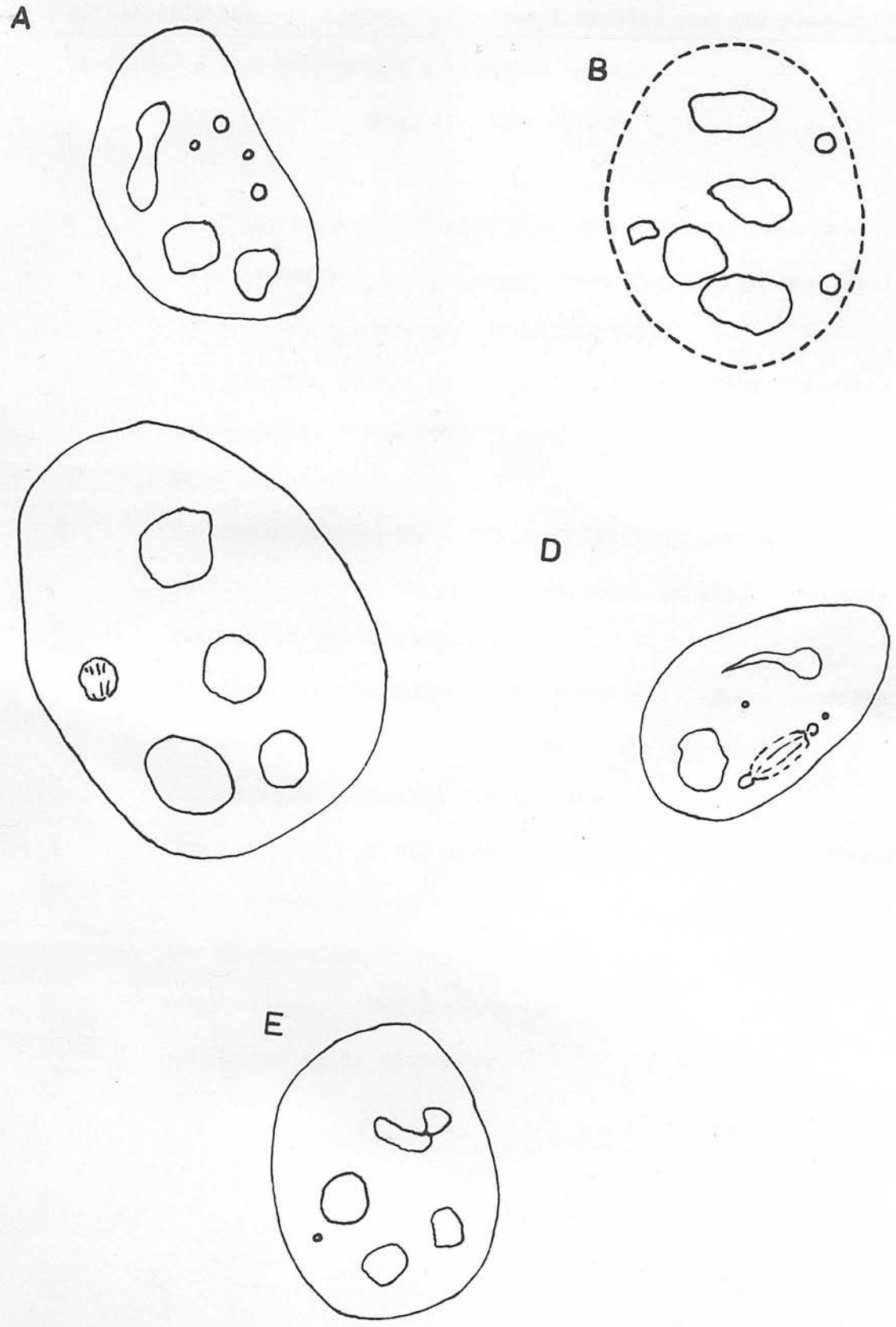

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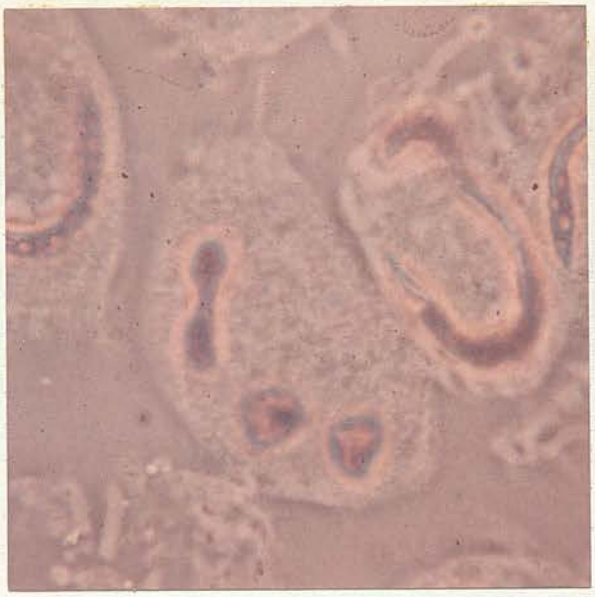
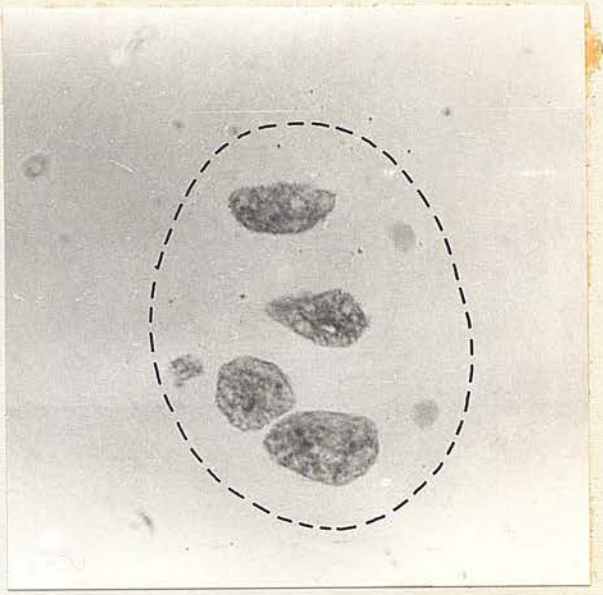
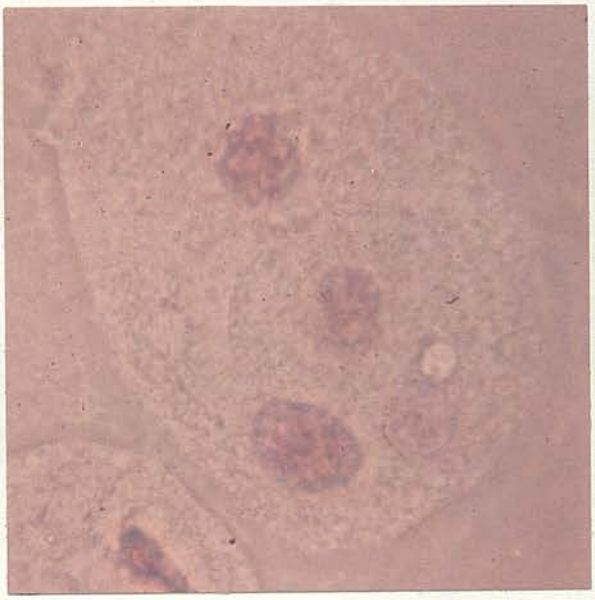
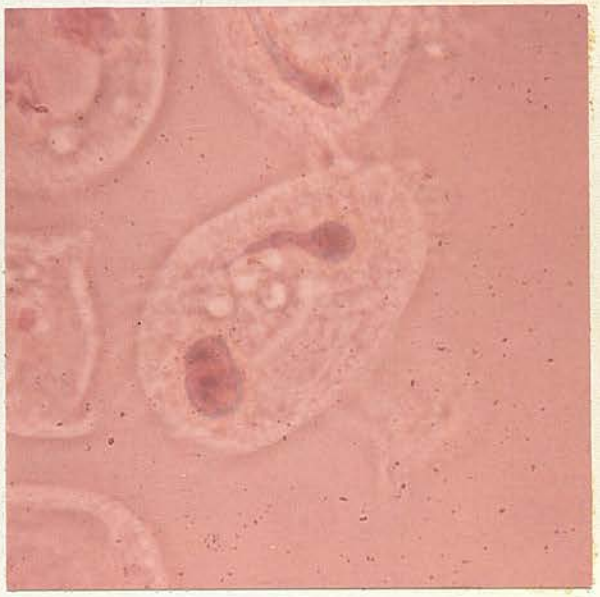
Plate 12









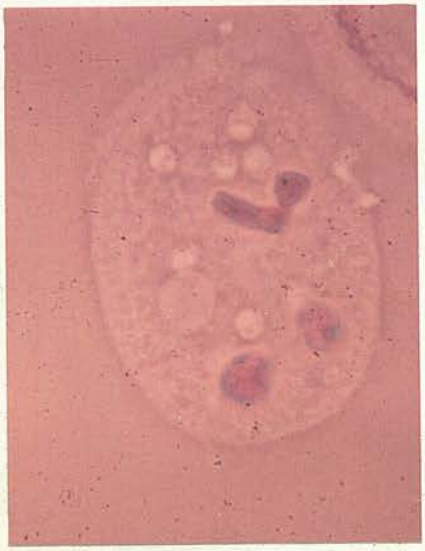


Plate 13

Growth of the macronuclear anlage and its eventual disurption, in Feulgen stained autogamous animals.

Magnification x 1050

A The macronuclear anlage has grown to 12μ and stains lightly. The posterior fragments of the old macronucleus continue to break up and disperse.

B The posterior fragments have vanished. The anlage has increased in stainability but is homogeneous.

C Small granules are present in the anlage, which now stains still more heavily.

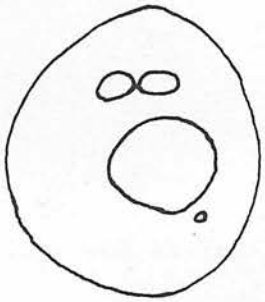
D The granules have coalesced into a spireme. The matrix of the anlage appears Feulgen negative.

E The spireme has been replaced by a condensed mass of Feulgen positive material, still within the membrane of the anlage. 2 old macronuclear fragments are still present.

F The anlage has broken and the contents are scattered in the cytoplasm. One old macronuclear fragment remains in this exautogamous animal, and it is beginning to change in appearance.

A

B



C

D

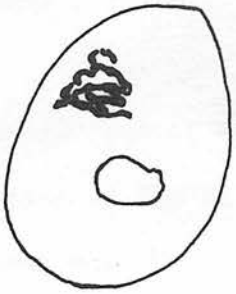


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Plate 13

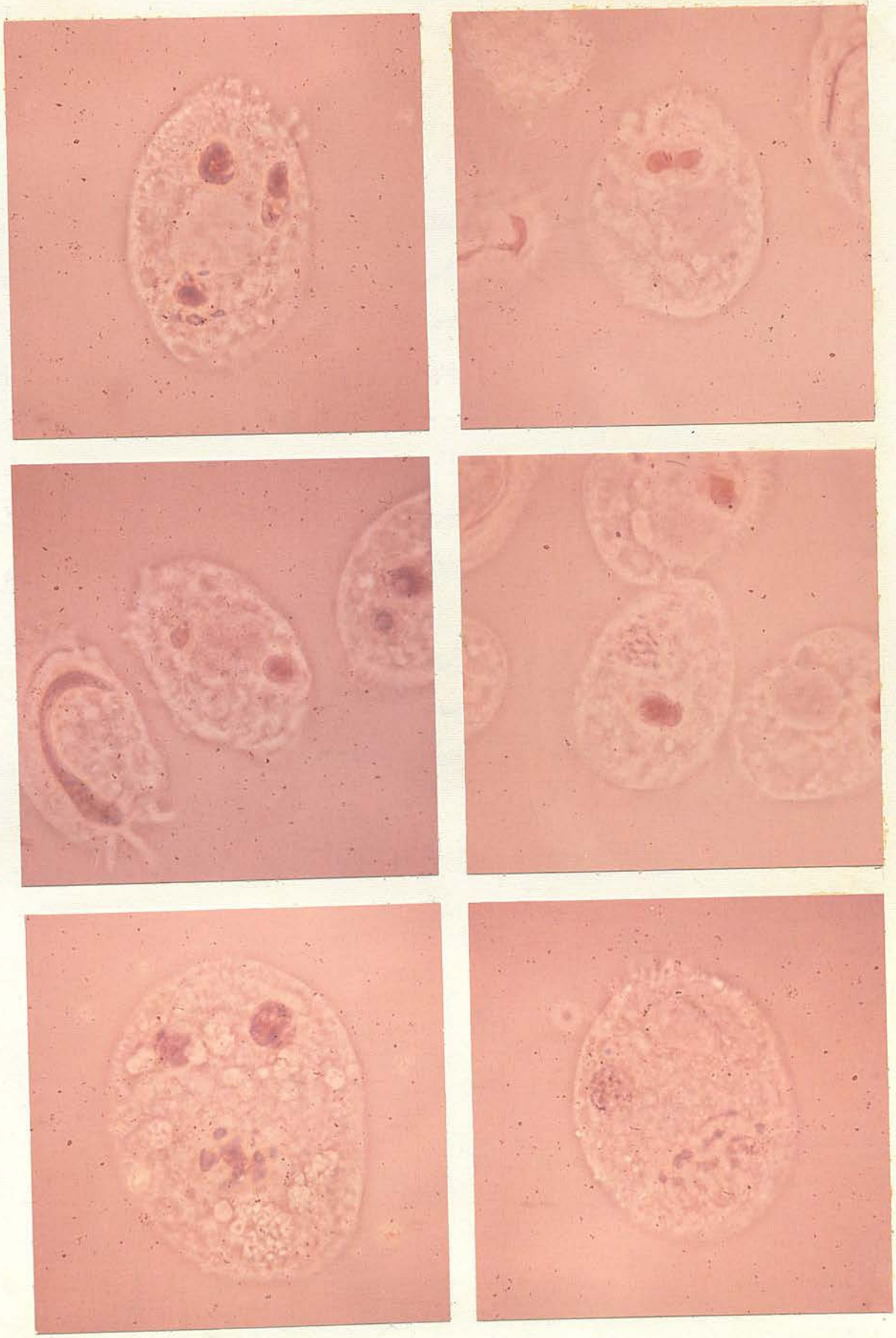

Plate 14

Macronuclear regeneration following disruption of the anlagen of exautogamous animals.

Magnification x 1050

A The contents of the anlage are scattered in the cytoplasm of an exautogamous animal. 2 fragments of the old macronucleus are changing in appearance.

B Exautogamous animal containing one regenerating fragment of the old macronucleus. No other Feulgen positive material is visible.

C The regenerating fragment has increased in size. It has a reticular appearance unlike that of the reorganising anlage of an exconjugant.

D The regenerating macronucleus is growing around the anterior margin of the exautogamous animal.

E The regenerating macronucleus then grows around the posterior margin.

F An exautogamous animal containing 2 regenerating fragments.

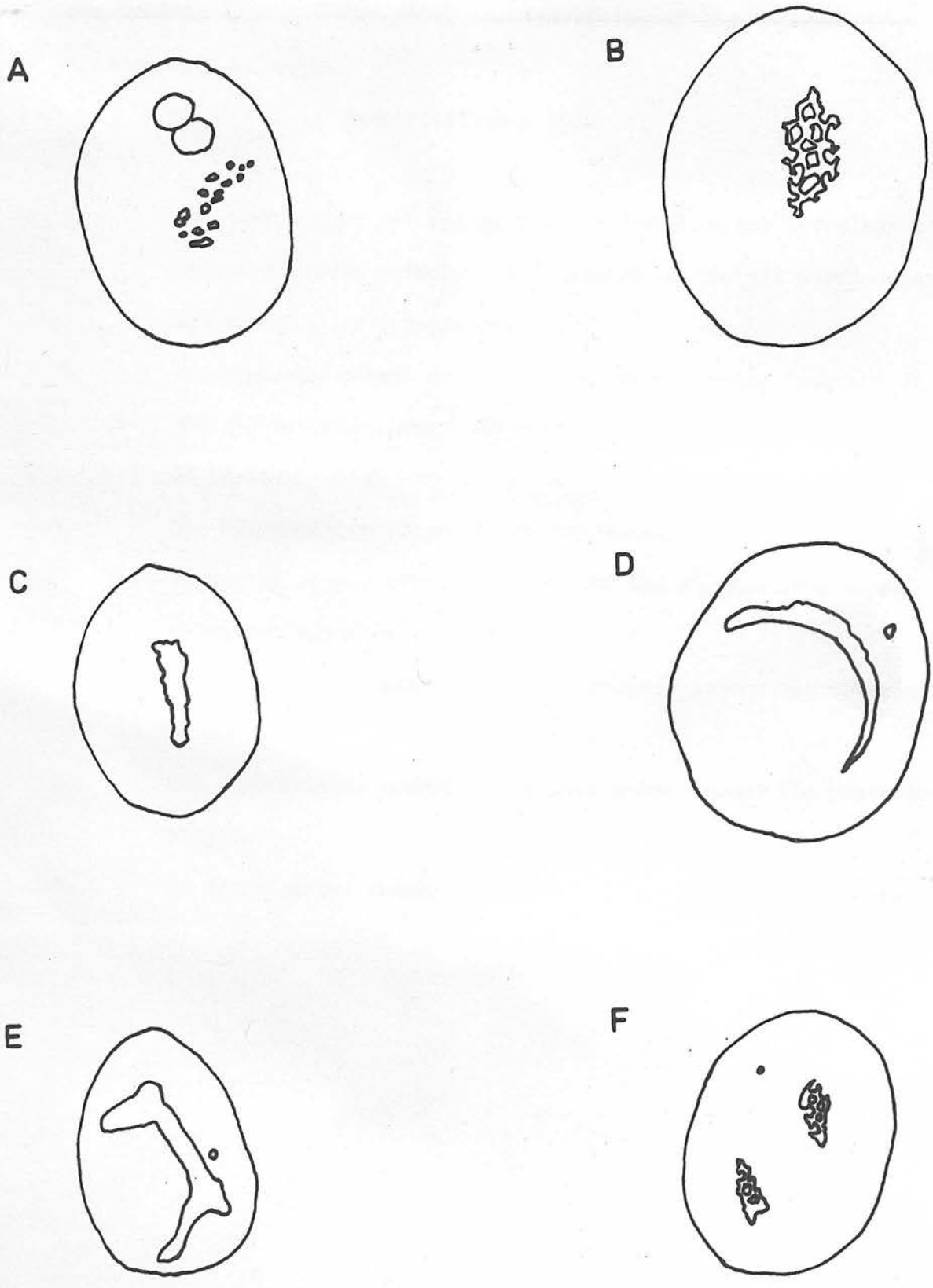
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Plate 14

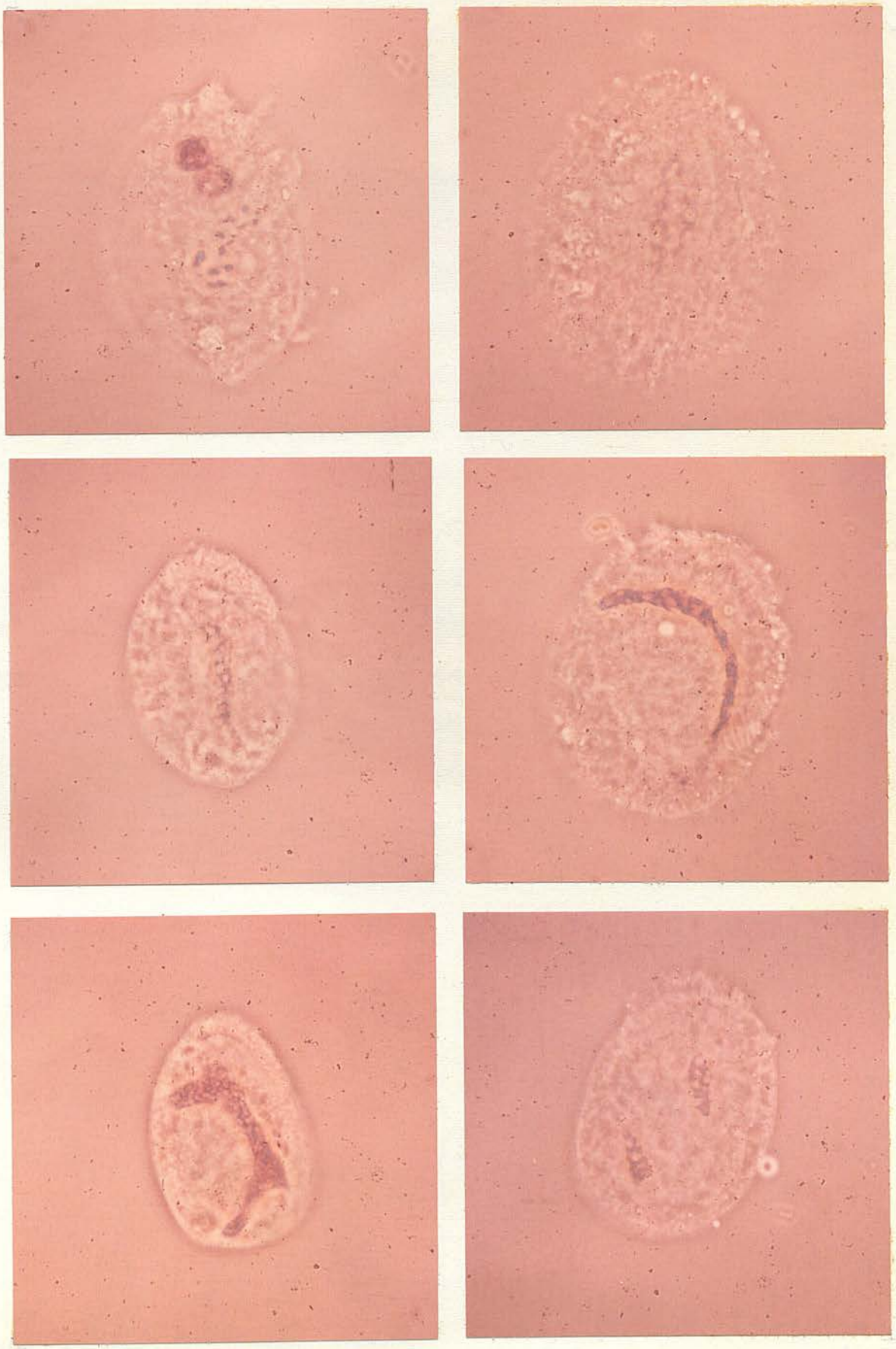

Plate 15

The growth of macronuclear fragments in exautogamous animals.

Magnification x 1050

A Exautogamous animsl with 2 regenerating fragments and a single micronucleus.

B The 2 fragments have grown in size.

C The 2 fragments may nearly touch. In this exautogamous animal the micronucleus lies between the 2 fragments.

D Unusual macronucleus seen in exautogamous animals, possibly caused by fusion of 2 fragments.

E Macronucleus of bizarre shape, possibly due to fusion of 2 regenerated fragments.

F Exautogamous animal with large diffuse macronucleus. Reorganisation bands are travelling from the tips towards the centre. The regions through which the bands have passed stain more heavily indicating their greater DNA content.

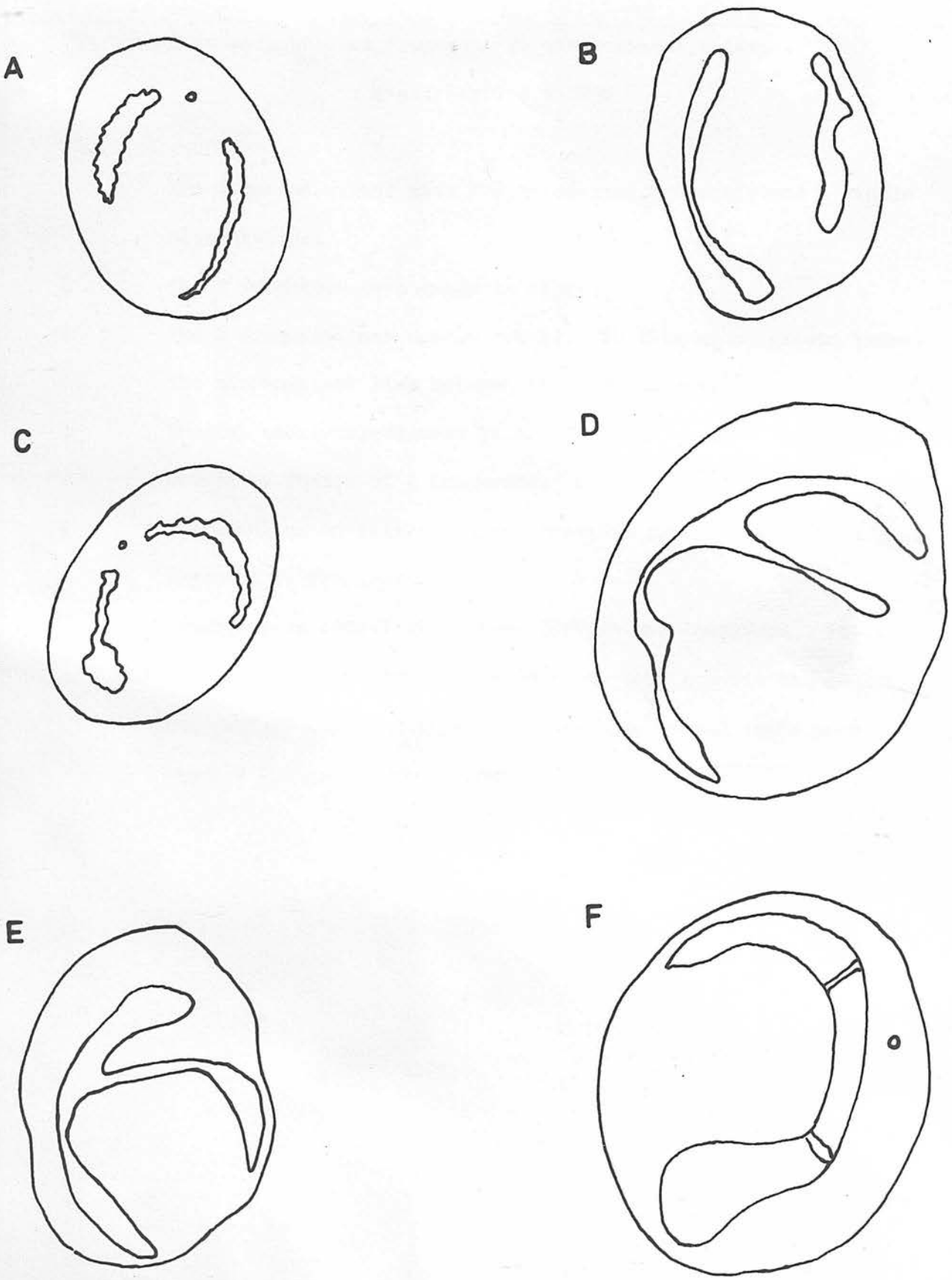
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Plate 15

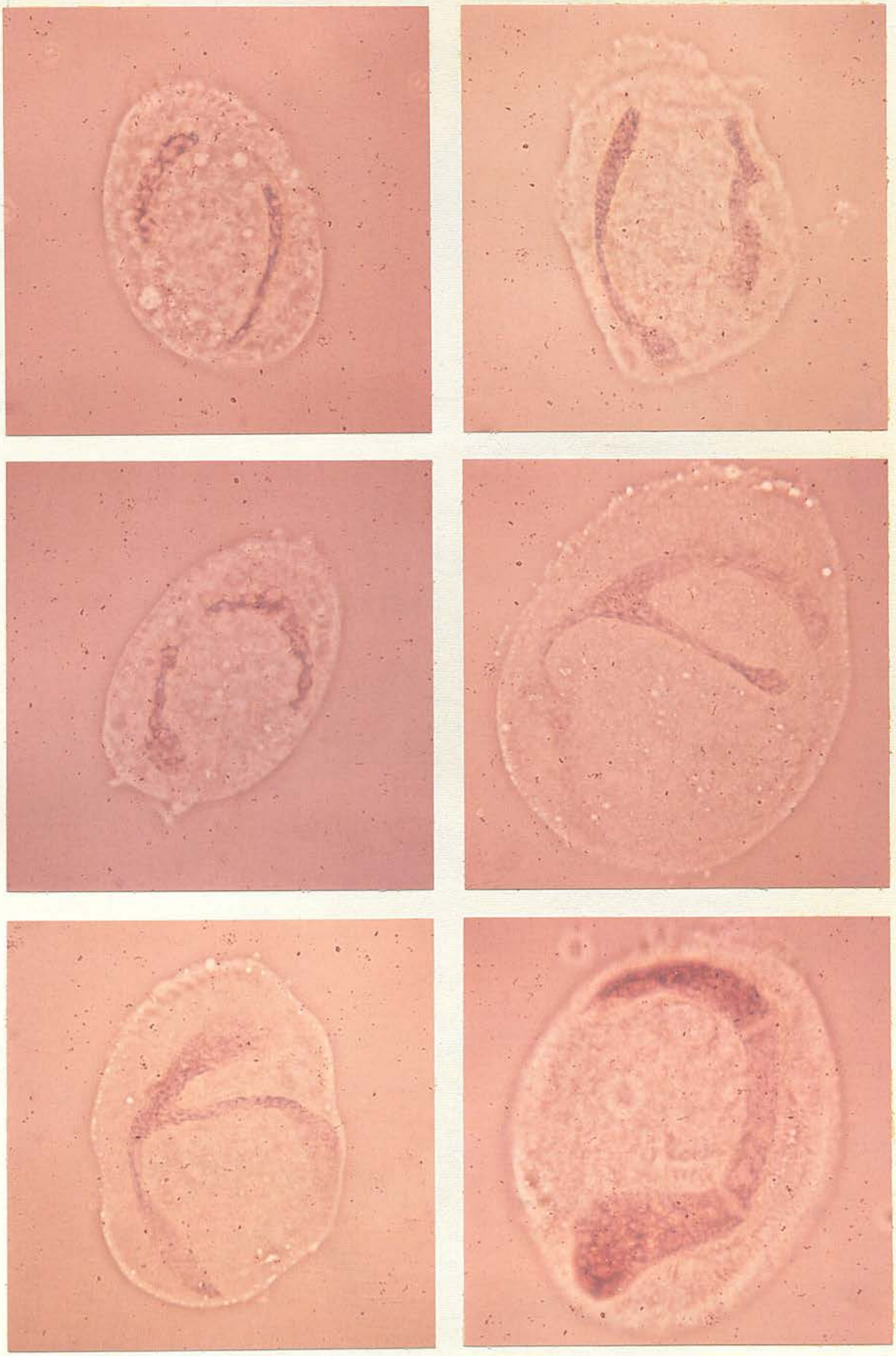


Plate 16

Rare events during autogamy.

Magnification x 1050

A Exautogamous animal in which the macronucleus has the appearance of the reorganising macronucleus of exconjugants. In this animal the macronucleus may be derived from the macronuclear anlage.

B Abnormal exautogamous animal with a single anlage, but 3 micronuclei. Such an abnormality could arise if a 2nd post-zygotic division had occurred.

C Abnormal exautogamous animal with 2 macronuclear anlagen, but no apparent micronucleus.

A

B

C

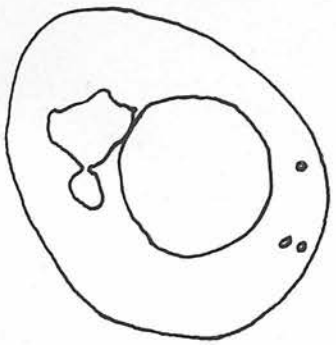


Plate 16

PART IV

Genotypic expectations after conjugation and autogamy in Euplotes minuta

Knowledge of the cytological events underlying conjugation and autogamy make it possible to compute the expectations for the various genotypes after fertilisation. However, considerable confusion has arisen over the calculation of the probabilities of survival of nuclei at the different stages which culminate in the production of gametes. This was first noted by Sonneborn who with Behme (1966) assessed the data available, and constructed new theoretical expectations. Let us adopt their nomenclature and consider a single locus (Aa) and the formation of gametes in a single cell.

Aa

STEP I	Preliminary division:	Aa	Aa
	Two meiotic divisions: (8 haploid nuclei)	(A) (A) (a) (a)	(A) (A) (a) (a)
	Two persist:	(A) and (A), or (A) and (a), or (a) and (a)	
STEP II	Each divides:	(A) (A) (A) (A), or (A) (A) (a) (a), or (a) (a) (a) (a)	
	Two become gametes:	(A) and (A)	or (A) and (A) or (A) and (a) or (a) and (a) (a) and (a)

Sonneborn and Behme consider that error arose firstly in connection with step I over calculation of the probability of survival of 2 out of the 8 haploid nuclei. It was originally assumed by Heckmann (1963,1964) that because there were equal numbers of (A) and (a) nuclei after the 2nd. meiotic division, the probability that an (A) nucleus would persist was 1/2. There was the same probability that an (a) nucleus would survive. Therefore the probability of

the two survivor nuclei being both (A) was 1/2 x 1/2 = 1/4; of both being (a) was 1/4, and of one being (A) and the other (a) was 1/2. The same error arose in calculating probabilities at step II. Hence it was incorrectly assumed by Heckmann that from a test cross of (Aa) to (aa), both exconjugants would have the genotype (Aa) in 1/4 of the pairs, both would be (aa) in 1/4 of the pairs, and in the remaining 1/2 one member of the pair would be (aa) and the other (Aa).

Sonneborn and Behme commented that if one (A) nucleus persists at step I the probabilities are no longer equal for the survival of (A) or (a) in the second nucleus, as there are 4(a), but only 3(A) nuclei remaining. There are in fact a total of 6 ways for two (A) nuclei to persist, likewise 6 ways for two (a) nuclei to persist, and 16 ways for the surviving nuclei to consist of one (A) and one (a) nucleus: 28 alternatives in all.

After the mitotic division of the survivor nuclei, 2 of the 4 pronuclei become the functional gametic nuclei (Sonneborn's step II). If the gametic nuclei are always sister nuclei, co-conjugants should always be alike with respect to genotype. Kimball's genetic study on E.patella (1942) showed that in this species the two members of a pair gave rise to lines of the same mating type in 63 out of 69 pairs. The remaining 6 pairs could have been derived by cytogamy, or may have been cases of non-sister nuclei becoming gametes. The cytological aspects of conjugation in this species (Katashima, 1960) confirm that sister nuclei normally give rise to gametes. Consideration of the outcome of crosses in E.crassus and E.vannus (Heckmann, 1963, 1964), and Cohen's early work on a species which he called E.patella (Cohen, 1934) (but Pierson (1943) later identified as E.eurystomus), suggested that there was no uniformity in whether the gametic nuclei arose from one or two different products of meiosis.

When the nuclei persisting at step I are of the same genotype, the gametic

nuclei must also be. In 6/28 conjugants both gametes are (A), according to Sonneborn and Behme's calculations, and in 6/28 conjugants both gametes are (a). But these two classes are augmented by members of the third class.

If the pronuclei are (A_1) (A_2) (a_1) (a_2) from the persistence of one (A) and one (a) nucleus after step I, and there is randomness as to which become gametes, then there is one way in which two (A)s can be gametes, but 4 ways in which one (A) can be combined with one (a), namely: (A_1 and a_1) (A_1 and a_2) (A_2 and a_1) and (A_2 and a_2). Therefore in 1/6 of the 16/28 cases in which one (A) and one (a) nuclei survive after step I, both gametic nuclei are (A), and this value must be added to the 6/28 above to give a total of 13/42 conjugants having both gametes of type (A). Similarly 13/42 conjugants would have both gametes of type (a). The remaining 16/42 will have one gamete (A) ~~gamete~~ and one (a). Thus, according to Sonneborn and Behme, in a test cross of (Aa) to (aa) the foregoing calculations yield:

both exconjugants (Aa) in 13/42 of the pairs,
both exconjugants (aa) in 13/42 of the pairs,
and one exconjugant (Aa) and the other (aa) in 16/42 of the pairs.

This means that the co-conjugants would be alike in 26/42 of the pairs, and unlike in 16/42 of the pairs; a ratio of 13:8 and not of 1:1 as previously predicted by Heckmann. The expected proportions for synkarya after autogamy under this scheme are 13(AA):16(Aa):13(aa). It was against these ratios that Nobili and Luporini (1967) tested their segregation data.

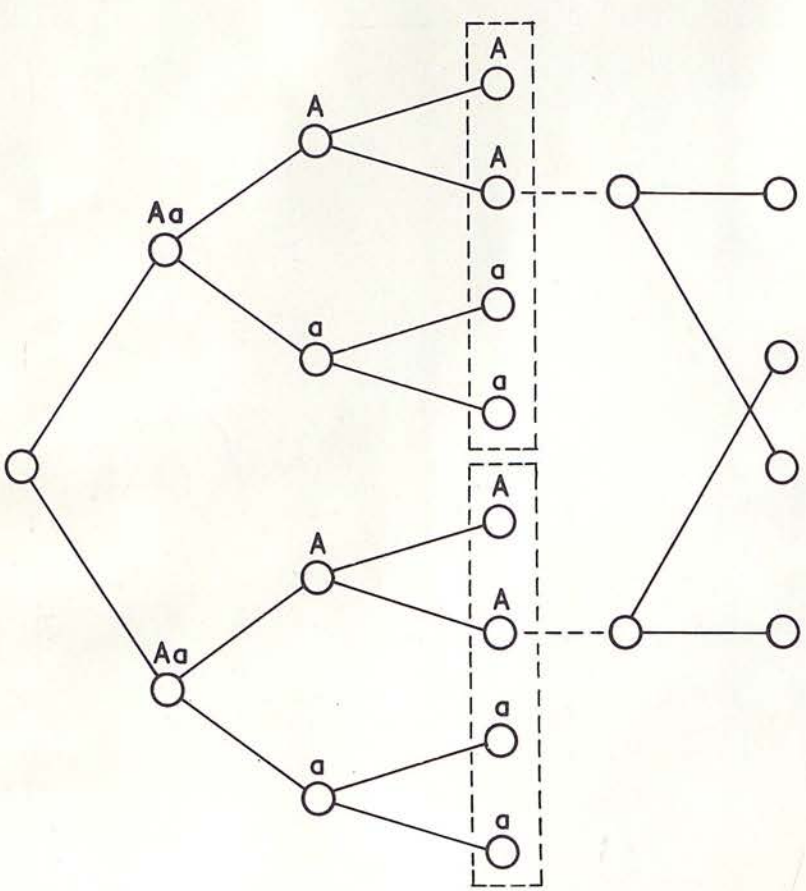
It is clear that the logic applied by Sonneborn and Behme in calculating the probabilities of survival of nuclei at step I, depends upon there being no restrictions on the choice of nuclei. In the light of cytological evidence provided by Katashima (1959a, 1960) and in this present study, we know that this is not true. All cytological studies on Euplotes have shown that one survivor

Figure 8.

Scheme of

nuclear behaviour in

E. minuta and E. cristatus

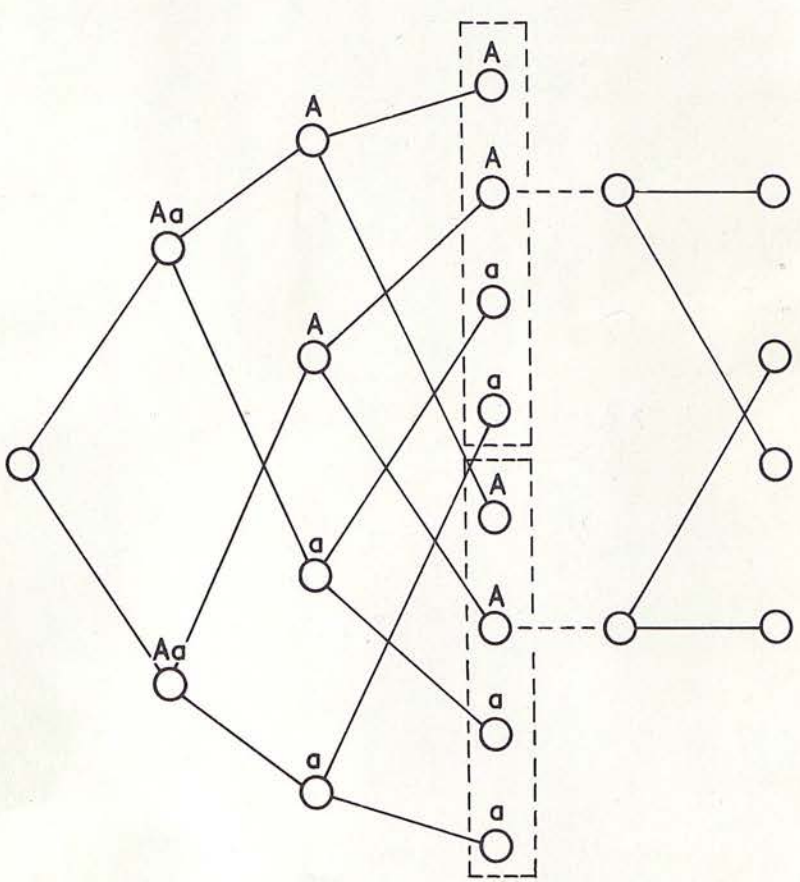


Scheme of

nuclear behaviour in

E. crassus, E. vannus, E. patella

and E. eurystomus



nucleus is chosen from an anterior group of 4 nuclei, the other survivor from the group of 4 nuclei posteriorly. Within a group of 4 nuclei the relationships of the haploid nuclei to each other may vary with the species. In the case of E.cristatus (Wichterman, 1967) and E.minuta the behaviour of the nuclei makes it very likely that each group of 4 represents the 4 products of a meiotic division. The persistence of one haploid nucleus from each group means that they are derived from separate meiotic events. Consequently the problem arising over the selection of 2 nuclei from 8 (where the choice of one influences the probability of survival of the others) is removed. The choice is now of one nucleus from the anterior set, and a choice of one from the posterior set, and the two events are quite independent. In this case with each set of 4 nuclei consisting of (A) (A) (a) and (a), the probability of there being two surviving (A) nuclei is 1/4, of two (a) nuclei 1/4, and an (A) and an (a) nucleus surviving is 1/2.

Does this also apply in the case of E.patella, E.eurystomus, E.vannus and E.crassus where it seems likely that spindles overlap according to the scheme in Figure 8? (We must also allow that although it has not been detected cytologically, a system of overlapping spindles could operate in E.minuta.) If there has been no cross-over between the 'A' locus and its centromere, each group of 4 haploid nuclei will consist of two (A) nuclei and two (a) nuclei. If there has been a cross-over between the centromere and the 'A' locus during the first meiotic division in either or both of the nuclei, this will result in a distribution of the genes, such that the anterior group of 4, may consist of nuclei carrying 0,1,2,3, or 4 (A) genes (and therefore 4,3,2,1 or 0 (a) genes). However if all possible combinations are summed it is found that every nucleus has an equal chance of being (A) or (a). Although each group of 4 is not the product of a single meiosis, again the choice of one nucleus from the anterior

group in no way affects the probability of choosing a nucleus from the posterior group carrying a particular gene. The probability of the survivor nuclei carrying two (A) genes is the same as for nuclei carrying two (a) genes, and is yet again 1/4. In the remaining 1/2 of cases, one nucleus will carry (A) and the other (a).

There may be more stringent control over the choice of nuclei for survival at step I as is suggested by Katashima's work (1960). He found that sister nuclei persist at step I in about 90% of cases. The alleles represented in the 4 pronuclei will then depend upon the cross-over frequency between the locus and the centromere. If the locus is very close to its centromere, sisters will usually be alike. The further the locus is from the centromere the greater the number of cases in which sisters will carry different alleles. A theoretical maximum exists at which 67% of sisters from the 2nd. meiotic division carry different alleles. However, if the gametic nuclei are always derived from the mitotic products of one of these survivor nuclei, one is unable to map centromere distances. In other species where there may be random selection of gametes from the 4 pronuclei, the survival of sister nuclei at step I need not imply that they carry the same alleles.

To summarise the arguments about step I, it seems probable that the ratio for survivor nuclei both carrying (A) genes: both carrying (a) : one carrying (A) and the other (a), is 1:1:2 rather than the 6:6:16 ratio suggested by Sonneborn and Behme. Let us now examine the consequences of step II starting with this 1:1:2 ratio, and attempt to predict the ratios expected in the gametes. We will again assume that gametes are chosen at random following mitosis of nuclei surviving at step I. In the conjugants which contained unlike survivors, the gametic nuclei will be both (A) in 1/6 of these conjugants, and both (a) in another 1/6, and these proportions must be added to the conjugants

Table 17

Tests of the observed ratios of pairs of conjugants that are alike to pairs that are unlike, against two different expectations.

Species	Observed Alike:Unlike	Expected 13:8	χ^2_1	p	Expected 2:1	χ^2_1	p
E.vannus	421:227	401.1:246.9	2.59	< 0.2	432:216	0.84	0.4
E.vannus	356:174	328.1:201.9	6.23	< 0.02	353.3:176.7	0.06	0.8
E.crassus	66:37	63.8:39.2	0.12	0.75	68.7:34.3	0.32	0.6
E.eurystomus	34:12	28.5:17.5	2.79	< 0.1	30.7:15.3	1.06	0.3
			$\chi^2_4 = 11.73, p=0.02$			$\chi^2_4=2.28, p=0.7$	
E.minuta	27:17	27.2:16.8	0.003	0.95	29.3:14.7	0.54	0.5
E.minuta	23:26	30.3:18.7	4.61	< 0.05	32.7:16.3	8.63	< 0.01

Sources of data: E.vannus, Heckmann, 1963. E. minuta (27:17), present study.
E.crassus, Heckmann, 1964. E. minuta (23:26), Nobili and Luporini, 1967.
E.eurystomus, Cohen, 1934.

which are obliged to produce gametes of these types. When 1/6 of 1/2 (1/12) is added to 1/4 we find that 1/3 of the conjugants possess gametic nuclei which are both (A). Similarly 1/3 have gametic nuclei which are both (a). In the final 1/3, one gametic nucleus is (A) and the other (a). In a test cross (Aa) x (aa):

both exconjugants will be (Aa) in 1/3 of the pairs,

both exconjugants will be (aa) in 1/3 of the pairs,

and one exconjugant will be (Aa) and the other (aa) in 1/3 of the pairs.

The two exconjugants should be alike in 2/3 of the pairs, and unlike in 1/3. The expected ratio when gametes combine at random after autogamy of a heterozygous clone, is therefore 1(AA): 1(Aa):1(aa). These ratios can clearly be modified if there are restrictions upon the choice of gametes, as we have seen in the case of E.patella (Kimball, 1942; Katashima, 1960).

We can examine the literature as Sonneborn has done, to see how closely the observed data from crosses of the kind (Aa) x (aa) fit with the ratio expected by Sonneborn and that proposed above. This comparison is made in Table 17. From such crosses Sonneborn and Behme's model, in which the choice of one nucleus for persistence at step I affects the choice of the second nucleus, predicts a ratio of pairs of conjugants that are alike:unlike of 13:8. The model proposed above, where the choice of two nuclei at step I is independent, predicts a ratio of 2:1. We can see immediately that Heckmann's data does not fit at all well the 1:1 ratio which he himself proposed. In the first four crosses in Table 17 the data were never significantly different from a 2:1 ratio whereas in two of the crosses the data did differ significantly from a 13:8 ratio. The four individual χ^2_1s obtained when testing the data against a 13:8 ratio were summed. The resulting $\chi^2_4 = 11.73$, and is significant at the 2% level (p=0.02) showing that the combined data can be confidently considered

not to fit the 13:8 ratio. When the four individual χ^2_1s obtained from testing the data against the 2:1 ratio were summed, the χ^2_4 obtained is definitely not significant (p=0.7), and the data as a whole thus fit a 2:1 ratio very well.

The deviation from the 13:8 model is consistently in the same direction, as alike pairs of conjugants always exceed expectation. To explain this, Sonneborn suggested that there is a slightly greater chance for gametic nuclei to be derived from the same meiotic product, or for two nuclei carrying the same allele to persist. When compared to a 2:1 ratio no consistent deviation is apparent: this model does not require non-random selection of gametic nuclei for synkaryon formation. One may conclude firstly that my model predicts more accurately than does Sonneborn's the ratio that will be seen after conjugation, and secondly that the combined data render Sonneborn's model very unlikely.

None of the three species which provided the data against which the two models have been tested, is autogamous. It is possible however in E.minuta to perform crosses with both autogamous and non-autogamous stocks. Crosses of the type (Aa) x (aa) appear to give different ratios of alike to unlike pairs of exconjugants, depending upon whether autogamous or non-autogamous stocks are used as parents.

In this present study a hybrid between an autogamous and a non-autogamous stock was backcrossed to the autogamous stock. 27 pairs recovered were alike in genotype and 17 differed. This ratio is not significantly different from a 2 : 1 ratio (p=0.5) and suggests that fusion of gametes is random, as in E.vannus, E.crassus and E.eurystomus.

Nobili and Luporini (1967) crossed an autogamous stock, heterozygous at the mating type locus, with a non-autogamous stock, and observed that 26 out of 49 pairs were unlike with respect to mating type. This 23 : 26 ratio is significantly different from a 2 : 1 ratio at the 1% level ($p < 0.01$). Although gametic nuclei could again be sisters or non-sisters, there was a

greater chance of selection of non-sisters.

In a third type of cross involving two non-autogamous parents, Nobili (1965, 1966) stated that only 10% of pairs had unlike nuclei. This would suggest that sister nuclei had a greatly increased chance of becoming gametes.

Is there some way in which we can reconcile these discrepancies in the genetic data? We will continue to assume that in a heterozygote both survivor nuclei at step I are (A) in 1/4 of the conjugants, both (a) in a further 1/4, and one is (A) and the other (a) in 1/2. It will be recalled that in the cytological study in Part III which involved two non-autogamous stocks, as did the third type of cross described above (Nobili, 1965, 1966) the percentage survival of the different nuclei at step II was calculated (see Table 16). It was found that spindles may or may not overlap during the 3rd pregamic division. If the spindles always overlapped, gametes would be sisters in 66% of conjugants, and non-sisters in 34%. However, because only 1/2 of the conjugants will have pronuclei differing in the alleles they carry, we can conclude that in only 17% of conjugants will gametes differ in genotype. Hence only 17% of pairs recovered after synkaryon formation will be unlike in genotype. 17% is the minimum percentage of unlike gametes which is at present consistent with the cytological data, and it approaches the 10% observed by Nobili (1965, 1966). To lower this minimum we would need to postulate that like nuclei survive at step I.

The cytological evidence in Part III showed that in 94% of cases one gametic nucleus is selected from the anterior two nuclei, and the other from the posterior pair. If there is usually no overlap of the spindles at the 3rd pregamic division, 47% of exconjugant pairs will differ in genotype. This approximates to the number of unlike pairs (26/49, or 53%) observed by Nobili and Luporini (1967) when an autogamous stock was used as the heterozygous parent.

It is therefore possible to explain the difference between the crosses by

Table 18

Tests of the observed ratios for several characters after autogamy in E. minuta, against two different expectations

	E. minuta	Observed (AA+Aa):(aa)	Expected 29:13	χ^2_1	p	Expected 2:1	χ^2_1	p
a)	mating type	70:1	49.0:22.0	27.68	< 0.001	47.3:23.7	31.21	< 0.001
		102:3	72.5:32.5	37.48		70.0:35.0	42.52	
b)	mating type	170:8	122.9:55.1	57.08		118.7:59.3	65.26	
	autogamy trait	161:17	122.9:55.1	37.16		118.7:59.3	44.18	
	temperature trait	153:25	122.9:55.1	23.13	< 0.001	118.7:59.3	28.89	< 0.001
	maximal corticotype	162:9	118.1:52.9	51.55		114.0:57.0	59.37	
c)	mating type	221:9	158.8:71.2	77.44	< 0.001	153.3:76.7	88.34	< 0.001
	esterase Es-1	113:3	80.1:35.9	42.35		77.3:38.7	48.15	

Sources of data:

a) Nobili and Luporini, (1967)

b) Heckmann and Frankel, (1968)

c) Present study

suggesting slight differences in the length of the nuclear spindles at the 3rd. pregamic division, so that these overlap in non-autogamous, but not in autogamous stocks. If a hybrid between these types of stock were intermediate, so that spindles overlapped in 50% of cases, a 2:1 ratio of alike to unlike pairs of conjugants, would be generated. There is no need to resort to differences in unidentified cytoplasmic factors which cause the gametic nuclei to differentiate.

Table 18 is a summary of the segregation ratios observed for various characters, after heterozygous stocks have undergone autogamy. Unfortunately, because of dominance, the homozygote (AA) has never been distinguished from the heterozygote (Aa), and segregation of the recessive homozygote (aa) only is observed. It is always assumed that the numbers of (AA) and (aa) homozygotes produced are the same. Following the arguments of Sonneborn and Behme, where choice of one of the 8 haploid nuclei after the 2nd. meiotic division affects the probability of chosing the other, the expected proportions of synkarya after autogamy are 13(AA):16(Aa):13(aa). With dominance this becomes a 29:13 ratio. Alternatively, if the choice of each survivor nucleus is independent, we conclude that the expectation for synkarya is 1(AA); 1(Aa): 1(aa) and when the (AA) and (Aa) genotypes are indistinguishable this becomes a 2:1 ratio. It is quite clear from Table 18 that neither expectation is fulfilled. If we assume that both homozygotes are present in equal frequencies, an enormous excess of heterozygotes must be producing the observed ratios. In other words, there is usually no change in phenotype following autogamy.

Nobili and Luporini (1967) have invoked preferential fusion of gametes carrying different alleles to explain the maintenance of heterozygosity after autogamy in E.minuta. They believed that the excess of unlike pairs observed in the cross already described, which involved an autogamous stock, supports this idea. It seems unreasonable to imagine that the two pronuclei

are selected on the basis of the particular alleles they carry at the mating type locus. A more feasible hypothesis would be that the two nuclei which form the gametes are more frequently non-sister nuclei, but this is a quite inadequate explanation of the observations. Fusion of sister nuclei would give rise to completely homozygous animals after autogamy. If synkaryon formation occurs randomly, 1/3 of all exautogamous clones will be totally homozygous. If we suppose that non-sisters usually fuse, these completely homozygous animals will be absent from the progeny and we can adapt our expectations accordingly. Remembering that nuclei persisting at step I will sometimes carry the same alleles Sonneborn and Behme's reasoning would give a ratio of 3(AA):8(Aa):3(aa) for the progeny of a heterozygous clone (Aa) after autogamy if gametes are usually non-sisters. The model in which the choices of survivors at step I are non-contingent, in this instance yields a ratio of 1 (AA):2(Aa):1(aa). Hence a maximum of 57% or 50% of exautogamous clones, according to the model adopted, would be heterozygous at any one locus. Whether or not these clones would also be heterozygous at other loci would depend upon linkage relationships.

Preferential fusion of non-sister nuclei, and likewise preferential selection of gametes carrying different alleles, are quite inadequate in explaining the maintenance of heterozygosity after autogamy, as it is clear from the results in Table 18 that heterozygosity is apparently retained at not just one locus, but at every locus studied. Other explanations requiring a selective advantage of heterozygotes are also unsatisfactory both for this reason and because selection in favour of heterozygotes would need to operate only after autogamy, and not after conjugation. (The ratios obtained after conjugation give no hint that such selection is acting.) As the behaviour of nuclei leading to synkaryon formation appears so similar at conjugation and autogamy, there is no straightforward Mendelian mechanism which will account for the very different ratios obtained after each. The cytological study in Part III indicated that the explanation for the maintenance of heterozygosity after

autogamy lies in the fact that macronuclear regeneration, which happens at a later stage, frequently occurs after autogamy but not after conjugation.

Conclusion

The genetic results failed to fulfil the theoretical expectations after autogamy. The cytological observations strongly suggested that the new macronucleus of most exautogamous animals is regenerated from the fragments of the old macronucleus, which persist in the cell. There is usually no change in the phenotype after autogamy.

For those characters which are definitely inherited in a simple Mendelian fashion, namely mating type and esterase Es-1, a change in genotype was observed in approximately 1 in 33 exautogamous clones. The exceptional clones were recessive homozygotes. A change in genotype which retains the dominant allele is not detected.

If we assume random fusion of gametes in hybrids, and my results suggested this does occur, we can expect that for every one recessive homozygote (aa) produced by genuine autogamy, there will be a heterozygote (Aa) and a dominant homozygote (AA) also produced. Out of 33 exautogamous clones on average 3 will have macronuclei derived from the division of the synkaryon and the remaining 30 will have macronuclei produced by regeneration.

Among the clones which are the products of a true autogamy there should be a measurable chance that a change in genotype at one locus will be associated with a detectable change in genotype at a second unlinked locus. One third of the recessive homozygotes at the first locus, should also be homozygous recessive at a second locus. Out of 116 clones examined not one was observed to be homozygous recessive at both the mating type and esterase Es-1 loci.
It will be necessary to examine larger numbers of truly autogamous clones in

order to test whether a change in genotype at one locus is associated with a change at another locus.

DISCUSSION

1. Macronuclear regeneration in ciliates

The polyploidy of the ciliate macronucleus, and the fact that remnants of the old macronucleus persist for some time after synkaryon formation has certain consequences in genetic studies of these organisms.

In particular, these two facts permit regeneration of a functional macronucleus from even a small macronuclear fragment. Although the consequences of macronuclear regeneration vary with the organism, in all cases the phenotype of the parental cell is retained.

It is worthwhile at this juncture to consider how widespread macronuclear regeneration is. It was Sonneborn (1940) who first discovered that in syngen 1 of Paramecium aurelia a small percentage of animals could develop a new macronucleus from a fragment of the old macronucleus, both at conjugation and autogamy. The same process was later described in animals of syngen 4 (Sonneborn, 1945). Exposure of paramecia to 38°C when the new macronucleus is normally differentiating inhibits, or delays, normal development of the anlage and enhances the number of macronuclear regenerate progeny. The frequency of such progeny is also increased in strains bearing the gene am (Sonneborn, 1954b; Nobili, 1961). Exconjugants and exautogamous animals of P.aurelia usually have two developing macronuclei, and two micronuclei, which are distributed between the two daughter cells at the first fission. The am gene controls unequal distribution of the nuclei at cell division, so that up to 30% of dividing cells can yield daughters with no new macronucleus, but containing fragments of the old one. Such cells undergo macronuclear regeneration.

With respect to macronuclear structure, the ability of any one of forty fragments to regenerate in P.aurelia indicates that the macronucleus is compounded of at least forty diploid genomes. Furthermore, macronuclear regeneration has provided a method of constructing heterocaryons, with different alleles in the

micronucleus, from those in the macronucleus (Sonneborn, 1946; 1954c). In this organism the macronucleus appears to hold complete control over the characters expressed; there is no evidence that micronuclear genes are active.

Although the supporting genetic evidence is lacking, macronuclear regeneration has been observed in Cyclophyra katharinae after experimental removal of the developing anlage (Kormos and Kormos, 1960); and in Paramecium woodruffi when exconjugants are transferred from room temperature to 27°C (Jankowski, 1961). At the higher temperature the rudiments of the new anlagen degenerate.

Seshachar and Dass (1953) also reported a process, which they called macronuclear regeneration, in Epistylis articulata. In this peritrich, upsets at binary fission can produce amacronucleate animals; the micronucleus may then divide and one product enlarge and assume the characteristic shape of the vegetative macronucleus. This phenomenon is similar to endomixis, which will be discussed shortly. It is not really comparable to macronuclear regeneration following sexual reproduction, as heterocaryons do not result.

Recently Cole and Siegel (1969) have given detailed genetic evidence for the ability of Paramecium bursaria to undergo macronuclear regeneration. In 1963 Siegel reported exceptional progeny in crosses of syngen 1 of this species. The mating type of an animal is determined by two unlinked loci in this species according to the scheme outlined below:

Mating Type	Genotype
I	AABB, AABb, AaBB, AaBb
II	aaBB, aaBb
III	aabb
IV	AAbb, Aabb

The original results established that in aberrant clones the genotypes of the macro- and micronuclei differed, and Siegel and Cole (1967) concluded that in the presence of an allele b^{-a}, there was an apparent loss of the allele B shortly after the two alleles were brought together. In their later work (Cole and Siegel, 1969) the 4 caryonides which resulted from the first division of both exconjugants from a cross AaBBTt x aabbTt, were examined in detail. Three of these were found to be homocaryotic, having genotypically identical macro- and micronuclei. At the mating type loci the genotype was AaBb; at the third locus where the allele T, dominant to t, determines temperature resistance, the genotype was tt in all three clones. In the fourth caryonide crosses revealed the micronuclear genotype also to be AaBbtt, but this cell line expressed mating type III, and presumably therefore had the macronuclear genotype aabb; it was also temperature resistant. This caryonide was evidently a heterocaryon at three loci, and had retained the macronuclear genotype of one of its parents. Cole and Siegel assumed that both mates must have formed fertilisation nuclei AaBbtt. Whereas in one, development was normal, in the other, two normal micronuclei were formed, but only one macronucleus. The caryonide which was without a macronucleus after the first fission, must have regenerated one from the parent macronucleus. In retrospect, aberrant clones previously explained by mutational changes at the B locus, can now be considered to have arisen by regeneration. They did not invoke this phenomenon before to explain embarrassing results in this species as the macronucleus appeared to be lost early in the conjugation process. Less than 5% of the progeny are exceptional, so that macronuclear regeneration is not at all frequent in P.bursaria.

Certain irregularities noticed by Allen (1963) and Nanney (1963) in Tetrahymena pyriformis are also of interest in this context. In crosses of inbred stocks C and B, almost 100% of the progeny appeared BB, and not BC. This phenomenon of 'genomic exclusion' affects both exconjugants of a pair, and the progeny are homozygous and diploid. Cytogenetic studies employing interrupted mating techniques showed that genomic exclusion involves two rounds of mating (Allen, 1967a,b). Stock C, which does not contribute any genes to the offspring has a defective micronucleus. The normal conjugant produces two haploid pronuclei from one meiotic product, and one is transferred to the defective conjugant. Diploidy is re-established in each cell by endoreduplication or by division of the haploid pronucleus and fusion of the two products. Two post-zygotic divisions follow, producing two macronuclear anlagen and two micronuclei - but the anlagen fail to become functional, and the parental macronucleus remains almost full-sized. Separation now occurs, and if the two conjugants are typed genetically they are found to be unlike and to have retained the phenotypes of the parents. However the defective micronucleus of the C strain has been replaced by a normal diploid nucleus.

A second round of mating usually follows the first, and shows that the conjugants from round 1 are indeed heterocaryons. Meiosis, exchange of nuclei, synkaryon formation and the post-zygotic divisions all follow the normal course. The parental macronucleus is now discarded. Genetic evidence proves both exconjugants to be alike, and homozygous for the genes of the normal parent.

Genomic exclusion appears to be prevalent in many stocks of syngen 1 with no micronucleus or a deficient one, and may have evolved to restore a functional micronucleus (Allen et al., 1967). The stimulus for the retention of the

parental macronucleus can only be guessed at. Allen (1967b) suggested that the step which makes the pronucleus diploid, and which renders genomic exclusion analogous to autogamy, induces the old macronucleus to regain its function. Although the term 'regeneration' has been used, it is clear that the parental macronucleus does not fragment, and that there is very little decrease in mass. Variations in enzyme profiles that occur in heterozygotes as the clones reach maturity are retained when the macronucleus is retained, after the first round of mating (Allen, 1965). It seems likely that very little degeneration occurs during the first phase of genomic exclusion. It is then perhaps not surprising that exconjugants from the first phase are sexually mature, and able to reconjugate immediately, although round 2 exconjugants have a characteristic immature period.

After conjugation between stocks of syngen 1 of P.aurelia there is normally a period of sexual immaturity. However lines which pass through macronuclear regeneration usually lack an immature period, or it is abnormally short (Siegel, 1961). On this evidence the presence of an immature period would therefore indicate normal development of a new macronuclear anlage from the division of the synkaryon, whereas macronuclear regeneration, or retention, could be characterised by immediate maturity. This association between macronuclear regeneration and the absence of an immature period does not appear to exist in E.minuta as a period of immaturity always follows autogamy. This species is not the only exception, however, as in P.aurelia where a new macronucleus is constructed there is no immaturity following autogamy, although cytologically the process seems so very similar to conjugation. There also exist some strains of P.aurelia and T.pyriformis which are mature immediately after conjugation. One must conclude that the expression of the mating type genes, which determine the onset of maturity is controlled within the macro-

nucleus and may be influenced by the nature of the events which cause the differentiation of a new macronucleus, whether by regeneration or from the products of the post-zygotic division.

Cole and Siegel (1969) tentatively suggested that macronuclear regeneration can explain anomalous results obtained by several ciliate geneticists. They quoted the results of crosses performed by Kimball (1942) using E.patella and Heckmann (1964) using E.crassus. From one cross Kimball recovered 10 exceptional progeny, 5 of each parental type. Heckmann observed 13 exceptional clones, which again resembled the parents. If Heckmann's earlier work on E.vannus (1963) is examined we find that a total of 16 clones do not conform with the expected phenotype of the F_1. Of these, 10 resembled one of the parents, and 6 were homozygous for a recessive allele present only in the heterozygous condition in one parent. These last 6 clones could reasonably have arisen by cytogamy. The total of 33 clones which resembled the parent could have arisen firstly from non-conjugants, secondly by cytogamy, or thirdly by macronuclear regeneration.

Provided the crosses are carefully performed non-conjugants should not appear. It is not an easy matter to distinguish between cytogamy and macronuclear regeneration, as most of the abnormal progeny are derived from crosses of the kind: $mt^5/mt^3 \times mt^1/mt^1$. Only abnormal behaviour of the mt^1/mt^1 parent will be detectable, and cytogamy and macronuclear regeneration in such a stock will have the same genetic consequences. In only 4 of Heckmann's crosses with E.vannus would cytogamy be detectable, and in fact it was observed in 2 crosses of the type : $mt^3/mt^2 \times mt^2/mt^1$, which provided the 6 aberrant clones just mentioned. (It will be obvious that in this cross regenerate clones will pass unnoticed.) If the frequency of anomalous clones were greater in those cases where they could be produced by both macronuclear regeneration and

cytogamy, than in those cases where they could only be produced by cytogamy, this would be an argument in favour of macronuclear regeneration as the origin of at least a percentage of anomalous clones. E.vannus furnishes no support for Cole and Siegel's thesis that regeneration may occur during mating, but crosses with E.crassus suggest that it can.

From crosses in this species in which aberrant clones could be produced either by regeneration or by cytogamy, in fact 12 out of 875 progeny were aberrant. In 5 crosses in which there was the possibility of detecting cytogamy there was no unequivocal evidence that it did occur. Only 1 clone out of 397 obtained in these crosses was of unusual phenotype, and this clone could again have been produced equally by cytogamy or macronuclear regeneration. Thus E:crassus provides some evidence that exceptional clones produced in crosses may arise by macronuclear regeneration. Perhaps the most convincing data to support the claim that regeneration may replace normal differentiation of the anlage has been inadvertently supplied by Nobili and Luporini (1967) in E.minuta. They established that their line of stock A25 has the genotype mt^{7}/mt^{46C}, yet in a cross of this stock with strain 5G, which is genetically mt^{11}/mt^{10} they obtained the following segregation of mating type in the F_1 :

	Mating types of progeny				
	VII	XI	X	46C	Unclassified
Observed	20	37	15	0	0

Although Nobili and Luporini stated that the results conformed with expectation, it is clear from the genotypes of the parents firstly that progeny of mating type VII should not appear in the F_1, and secondly that the 1:1 ratio expected for mating types XI:X was not observed. If we assume that animals of mating type X are

the result of a genuine cross, then we would expect an approximately equal number of animals of mating type XI. If this is the case then about 40 or so clones (20 of mating type VII and about 22 of mating type XI) must have arisen by macronuclear regeneration or from non-conjugants. Cytogamy would have the effect of producing one clone of 46C mating type for every two expressing mating type VII, and one clone of mating type X for every two of mating type XI. Such ratios would be superimposed upon the 1 : 1 ratio obtained from genuine exconjugants. The presence of 20 clones expressing mating type VII, but lack of any of mating type 46C rules out cytogamy as an explanation of this aberrant ratio. It is less easy to eliminate non-conjugation as the source of the mating type VII, and excess of mating type XI clones. However the methods these workers employed adequately excluded non-conjugants in other crosses, so it is reasonable to assume that they would do so in this instance. We conclude, therefore, that the most likely origin of the aberrant clones is through macronuclear regeneration, although non-conjugation cannot be entirely discounted.

Examination of the literature on ciliate genetics can provide well proven examples of macronuclear regeneration in P.aurelia, P.bursaria and transient macronuclear retention in T.pyriformis; but normally only at a low frequency. Its occurrence in the genus Euplotes is not in itself surprising. There is evidence that macronuclear regeneration occurs normally at a low frequency during conjugation,but the peculiar feature in E.minuta is the high frequency at which it must occur to explain the maintanance of the parental phenotype after autogamy.

2. The heterocaryotic nature of exautogamous clones of E.minuta.

The ultimate proof for macronuclear regeneration would be a demonstration that exautogamous clones of E.minuta are heterocaryons. There is a limited amount of evidence that argues against the heterocaryotic nature of most exautogamous animals. Nobili and Luporini (1967) took animals of micronuclear genotype mt^{7}/mt^{46C} and allowed them to undergo autogamy. Out of 71 exautogamous clones, 1 expressed the 46C mating type and the others resembled the parent in expressing mating type VII. 12 of these last mentioned clones were crossed to a stock of genotype mt^{1}/mt^{1}. 50% of the progeny of each cross were phenotypically mating type VII, the other 50% were mating type 46C. The micronuclei of the 12 exautogamous clones were seemingly all heterozygous at this locus, whereas if the genotype of the micronucleus had changed after autogamy, despite the retention of the old macronucleus by regeneration, the crosses should have demonstrated micronuclear genotypes in the ratio $1mt^{7}/mt^{7}$: $1mt^{7}/mt^{46C}$: $1mt^{46C}/mt^{46C}$, after the reasoning presented in Part IV. There is a further brief report (Luporini and Nobili, 1967b) that hybrids between stock A31 and a stock of mating type I, behaved in a similar way. 16 exautogamous lines were backcrossed to the stock of genotype mt^{1}/mt^{1}. One line produced progeny which all expressed mating type A31, and the micronucleus of this line must have been homozygous for the mating type A31 allele. The other 15 clones produced progeny of both mating type A31 and mating type I, in a 1:1 ratio. Hence following autogamy of clones of genotype mt^{A31}/mt^{1}, the micronucleus retained this genotype in 15 out of 16 cases.

How could the genotype of both micronucleus and macronucleus remain unchanged after autogamy? One way out of this dilemma would be to propose that when the new macronuclear anlage disintegrates, and a fragment of the old macronucleus takes over, a similar process occurs with respect to the micronucleus. The cytological data would not be inconsistent with the replacement of the micro-

nucleus by a small fragment of the parental macronucleus containing a diploid genome. Failure to replace the micronucleus would produce amicronucleate clones. These we know do occur after autogamy, but could of course, arise in many ways. The only two descriptions of the formation of micronuclei from macronuclear fragments come from Schwartz (1958) who claimed micronuclei were formed in amicronucleate lines of P.bursaria from small macronuclear fragments; and secondly Ammermann (1970) who observed that 4 out of 14 amicronucleate clones of Stylonychia mytilus developed pseudomicronuclei, which resembled micronuclei in appearance, but could not replace them in conjugation.

Alternatively the parental genotype could persist in both macro- and micronuclei after autogamy if endomixis occurred. Endomixis has been defined by Raikov (1969) as the transformation of a micronucleus into a polyploid macronucleus without meiosis and karyogamy. In its simplest form it has already been described for Epistylis articulata (Seshachar and Dass, 1953). Diller (1928) discovered a more complex version of this process in the parasitic peritrich Trichodina. The macronucleus in this organism can degenerate and the single micronucleus then divide three times. One of the resulting nuclei becomes the new micronucleus and the other 7 enlarge into macronuclear anlagen which are distributed to daughter cells until there is one in every cell. There is a striking resemblance between this asexual process and conjugation, when 7 macronuclear anlagen are also formed in peritrichs. There is certainly no definite case of endomixis in the genus Euplotes although the observations of Möbius (1888) of many micronuclei, and of macronuclear fragments in cysts of E.harpa, just as they may suggest autogamy, could equally well be indicative of endomixis. The same is true of the reorganisation processes reported by Klee (1926), and Ivanić (1929a).

During autogamy in E.minuta there is no doubt that meiosis occurs. The

first meiotic division, with visible chromosomes, is quite distinct from the other divisions. Haploid nuclei are therefore formed and the diploid number restored by the fusion of two such products. The nuclear divisions are therefore not merely an asexual perpetuation of a pre-existing genome, as is the case with endomixis, evolved perhaps to replace a deficient macronucleus by a new one without change in genotype. All the cytological evidence suggests that during autogamy in E.minuta a genetically new macronuclear anlage, and a micronucleus with this same genotype, are formed, but for some reason as yet unknown, the anlage rarely becomes functional. The precise origin of the micronucleus in exautogamous clones is still debatable.

One scrap of data which can be interpreted as favouring the heterocaryotic nature of exautogamous clones, is the observation that the sub-line of stock A25 used in this study was found, from crosses, to have the micronuclear genotype mt^7/mt^7. Nobili and Luporini (1967) however have shown their line of this stock to be heterozygous- mt^7/mt^{46C}. It should be mentioned that the phenotypes of both lines, which are of course determined by the macronucleus, are indistinguishable. This may be because the two lines are indeed identical in this respect. My sub-line of stock A25 was derived from Nobili's stock by autogamy. Nobili and Luporini (1967) showed that the rare recessive homozygote mt^{46C}/mt^{46C} appeared in only 1 out of 71 exautogamous clones. The 1 clone phenotypically of this kind also carried the same alleles in the micronucleus. As already mentioned these workers believed that the majority of exautogamous clones have a micronuclear genotype mt^7/mt^{46C}. If the mt^7/mt^7 homozygote segregated at a frequency equal to the recessive homozygote there is a very remote chance (1-2%) of picking a clone of this kind to perpetuate the stock. If however autogamy results in micronuclear ratios of 1 mt^{46C}/mt^{46C} : 1 mt^7/mt^{46C} : 1 mt^7/mt^7 there is a probability of 33% of selecting a sub-line with a micronuclear genotype mt^7/mt^7 to start the next generation. It therefore seems far

more probable that my mt^7/mt^7 subline was derived by a change in the micronucleus rather than in both macro- and micronuclei. I would suggest that this rather slight evidence infers that a change in micronuclear genotype may be more frequent than a change in macronuclear genotype If such a situation exists a large proportion of exautogamous clones will be heterocaryons.

Any character which is affected by the micronucleus as well as the macronucleus, might well appear to be inherited in a simple Mendelian way at conjugation, but after autogamy its "genetics" might be very different from those of a character such as Es-1 which is probably controlled solely by the macronucleus.

We now know that when an F_1 clone is heterozygous at the esterase or mating-type loci, the recessive homozygote appears in the F_2 from autogamy at a frequency of around 1 in 33; this then is the frequency at which the macronucleus is replaced by one carrying only the recessive alleles. If the exautogamous clones are heterocaryons, having a macronucleus derived by regeneration but with a micronucleus which is the product of a genuine autogamy, how would we expect a character such as the ability to undergo autogamy to be inherited after autogamy?

Let us first examine the nature of this character. When an animal starts to undergo autogamy, the first visible change is a mitotic division of the micronucleus without cell division. Not until this has occurred is there a fragmentation of the macronucleus. Amicronucleate stocks, which are otherwise genetically competent to undergo autogamy, are unable to do so possibly because the initial mitotic division is necessary to trigger macronuclear breakdown. We cannot say definitely whether the ability of the micronucleus to enter this premeiotic division is a function of the genes in the micronucleus, or the macronucleus, or of the genes in both, but it is possible that the genotype of the micronucleus is significant. Heterocaryons having the macronucleus of an autogamous stock, but micronuclei which do not carry genes for autogamy, might well be unable to undergo autogamy.

This character appears in crosses to be controlled by a pair of alleles, the allele conferring autogamy being dominant over the allele present in the non-autogamous stocks. With random fusion of gametes during autogamy of an F_1 heterozygous at this locus, we expect 1/3 of exautogamous clones to have micronuclei bearing only the allele which does not permit autogamy; but we observe that only 1/33 of the exautogamous clones have macronuclei of this genotype.

We can formulate our expectations on this basis. If the genotype of the macronucleus is the important factor in determining the ability to express the autogamy trait, then 1/33 of exautogamous clones will be non-autogamous. If it is the genes in the micronucleus that matter, then 1/3 of exautogamous clones will be non-autogamous. If both sets of genes interact, then some fraction between 1/33 and 1/3 will lack the ability to undergo autogamy, and this is indeed what we observe for this character. Reference to Heckmann and Frankel's data (1968) in Table 18 shows that 1/10 of F_2 clones were non-autogamous, twice the number of recessive homozygotes observed for the mating type locus.

Temperature resistance is another character where the genotype of the micronucleus may be important in determining the phenotype of a clone. Temperature sensitive animals die within 48 hours of their transfer from 23°C to 28°C (Siegel and Heckmann, 1966; Heckmann and Frankel, 1968). At 28°C the cells fail to divide, they become quiescent and finally disintegrate. In crosses temperature sensitivity appears to be controlled by a single locus, the allele for sensitivity being recessive to the allele conferring temperature resistance. Sensitive clones are similar to amicronucleate clones in that the normal rate of division is greatly reduced, or even arrested. It seems very probable, therefore, that the genotype of the micronucleus plays some part in

determining the sensitivity or resistance of a cell. When the micronucleus carries only temperature sensitive genes it may be unable to divide at 28°C, regardless of the genes in the macronucleus. Assuming that exautogamous clones are heterocaryons and that the genotype of the micronucleus is important in this character, the number of sensitive clones recovered after autogamy of a line heterozygous at this locus should lie between 1/33 and 1/3 of all exautogamous clones. The ratio actually observed by Heckmann and Frankel (1968) is seen from Table 18 to be 1/7. Thus for both the autogamy and temperature traits there is a significantly higher number of segregants observed than at either the mating type or Es-1 loci.

We can turn this argument around and use it to say that the differences we observe in the number of segregants for several characters following autogamy, is consistent with the heterocaryotic nature of exautogamous clones. The characters which may be expressed, at least partially, through the activity of the micronucleus are those for which most change is observed after autogamy.

The evidence in favour of heterocaryons arising after autogamy in E.minuta is slim, and very indirect. A more extensive breeding programme is necessary before it can be decided whether or not the genes in the macronucleus are shared by the micronucleus.

3. Macronuclear regeneration as a normal occurrence in E.minuta, and its function.

Having established from both the cytological and the genetical viewpoint that macronuclear regeneration is very frequent during autogamy in E.minuta, we can ask certain questions about its occurrence and function. Firstly, is regeneration likely to occur in the wild, or are the observations made in this study the outcome of some selective procedure that has been employed in the laboratory? Both exconjugants and exautogamous animals initially look

identical, both superficially and cytologically. Both types of animal are quiescent while the surface structures are being reorganised. Such animals have been subjected to the same treatment. If the methods used favoured regeneration, both types of reorganising animals should behave in the same way, but they do not. It is not impossible that conditions may exist that support reorganisation of the anlage into a functional macronucleus in both types of animal, but they have not been found by Nobili or Heckmann and their co-workers, or myself, although variations in the temperature, the medium, and the time at which the animals are isolated have been used. In addition, although the percentage survival may vary, the ratio of macronuclear regeneration to genuine autogamy remains rather constant.

Of necessity the genetic studies must be performed with heterozygous animals. As within the autogamous stocks there is considerable uniformity for most characters available, hybrids between autogamous stocks and non-autogamous stocks have been used. Luporini and Nobili (1967a) have argued that the high mortality rate observed in the F_2 from autogamy of such hybrids, and the low survival in backcrosses, suggests that these two types of stock are reproductively isolated in the wild, and indeed my findings that most autogamous stocks share the same serotype and carry only Es-1^a alleles, supports their thesis that the two types of stock belong to different syngens. The possibility that there is some sort of genetic incompatibility, or deleterious combination of genes, in the macronuclei of F_2 clones can be eliminated. This is clear firstly from the cytological demonstration that macronuclear regeneration is not limited to hybrid clones, but is a normal feature of autogamy in the parental stock A25; and secondly from the data from crosses which show that backcross clones, and an F_2 obtained by crossing two F_1 clones, are quite able to produce functional macronuclei from their anlagen.

What evidence we have suggests that macronuclear regeneration is not

artificially produced either by the conditions used, or by the effect of crossing stocks which do not interbreed in the wild. Whether all autogamous stocks undergo regeneration we do not know. Certainly hybrids derived from stock A23 (Heckmann and Frankel, 1968) behave genetically as if regeneration were a characteristic of this autogamous stock also.

A second question we can ask is what advantage is there in replacing genuine autogamy by macronuclear regeneration? Perhaps we should firstly consider the consequences of a true autogamy, and for this we must refer to Paramecium aurelia. In this species autogamy produces animals which are totally homozygous (Sonneborn, 1939a,b) and mature, and at least some lines have an increased fission rate. The rejuvenating effect of autogamy is most apparent in the autogamous offspring of ageing parents. Sonneborn's observations show a "decline in fission rate in the absence of autogamy, and maintenance of high fission rate with recurrent autogamy" (Sonneborn, 1954a).

In the genetical sense autogamy in P.aurelia should be a dead end. After the process has occurred once, no further genetic changes by recombination or crossing over can occur at later autogamies. One must assume that the disadvantages of homozygosity, such as death due to recessive lethals and reduction in versatility, are outweighed by other benefits which are not purely the result of genes in new combinations. Such an idea has been proposed by Beale (1954).

A true autogamy in E.minuta would render 2/3 of all heterozygous loci, homozygous. (After 5 autogamies more than 99.5% of loci would be in the homozygous condition.) Macronuclear regeneration, on the other hand, retains in the active macronucleus maximum heterozygosity. Following either true autogamy or macronuclear regeneration E.minuta, unlike P.aurelia, is immature. Evidence for any rejuvenating effect of regeneration in E.minuta is indirect. Of the F_2 clones from autogamy which were typed for the characters studied, the

clones in which segregation was observed, and which must therefore have been derived by a true autogamy, grew no faster than those which had presumably undergone macronuclear regeneration. In those stocks where no nuclear reorganisation process is possible, fission rates fall with age. During the period of this study 5 out of 8 non-autogamous stocks were lost apparently due to declining vigour. In contrast, only 1 out of 6 of the stocks capable of undergoing periodic macronuclear reorganisation was lost. If however these stocks are prevented from undergoing autogamy they also became senescent (Luporini, unpublished). Because of the extremely low frequency with which an old macronucleus is replaced from the products of the synkaryon, the maintenance of a high fission rate in these stocks must be due to their ability to undergo macronuclear regeneration.

We can conclude that autogamy in P.aurelia, and macronuclear regeneration in E.minuta are both rejuvenating processes. In addition, a method of counteracting the effects of homozygosity appears to have evolved in E.minuta. By preserving as many loci as possible in the heterozygous condition the organism is potentially more versatile in coping with diverse environments. This mechanism is useless, unless heterozygosity is initially generated by mating, and it follows that after conjugation the macronucleus must be formed anew from the products of the fertilisation nucleus, and indeed regeneration appears to occur only rarely when autogamous, or other stocks, mate.

The nature of the stimuli which cause exconjugants to develop a macronucleus from an anlage, but autogamous animals to form a new macronucleus by regeneration is quite unknown. The similarity in the cytological events preceding gamete formation, suggests they play no part in determining the origin of the new macronucleus. The most obvious difference between conjugation and autogamy is the presence of a co-mate during conjugation. If it is the presence or absence of a mate which can contribute to the fertilisation nucleus that is the

important factor, then the method by which a new macronucleus is to be formed is already programmed in the cell, several days before the event. A partial test of this idea would be a clear demonstration that when a mate fails to contribute to a cross that it is indeed macronuclear regeneration that occurs, as the tentative evidence presented has already suggested. It will also be remembered that in Tetrahymena pyriformis, when a mate is unable to produce functional pronuclei, the macronucleus is retained in those conjugants which produce synkarya by a process resembling autogamy.

4. Macronuclear regeneration and theories of ageing

The fact that both regeneration in E.minuta and autogamy in P.aurelia prevent the effects of ageing sheds some light upon the nature of the rejuvenating process. Primarily this means that rejuvenation is less likely to be the result of elimination of incomplete genomes, or of harmful genes accumulated during the interautogamous period. If it was these that caused senescence, regeneration in E.minuta would be very inefficient at eliminating them. Let us consider this point in more detail. Ringertz et al. (1967) have estimated that the DNA content of the macronucleus of Euplotes eurystomus is 100-120 times greater than the DNA content of the micronucleus. The macronucleus is therefore about 200-240 ploid. We can also estimate that the fragment which regenerates at autogamy in E.minuta is 1/6th to 1/8th the size of the original macronucleus. Also, of course, in a considerable number of cases 2 such fragments regenerate. Applying these values we can calculate that if one fragment gives rise to a new macronucleus it will already be polyploid to the extent of 25-40N; or if two such fragments begin to regenerate the new macronucleus starts with a ploidy of 50-80N. So plainly there is only limited scope for the regulation of the balance of the genome by elimination of genomes that are aneuploid, chromosomes in excess, or of

deleterious genes. If we are to retain Faure-Fremiet's hypothesis (1953) that it is chromosomal imbalance which causes senescence, it must assume rather a different meaning.

Other workers have rejected the theory that senescence is due to chromosome inequalities on the grounds that alleles do not segregate in ageing heterozygous clones of P.aurelia, even after macronuclear regeneration (Sonneborn and Schneller, 1955; Sonneborn et al., 1956). Similarly the observation that high doses of X-irradiation fail to cause the reduced fission rate seen in ageing cultures of P.aurelia led Kimball and Gaither (1954) to conclude that senescence is not due to macronuclear mutation.

Perhaps rejuvenation is the manifestation of a new cycle of macronuclear gene activity. Chromosomes and genes may be available for control purposes only at certain times. In E.minuta the time at which controlling histones, or other proteins, or nucleic acids have access to their targets may correspond to the initial elongation of the macronuclear anlage in exconjugants and truly exautogamous animals. Certainly before this time there is no evidence that any transcription of RNA occurs in hypotrichs (Ammermann, 1965, 1968; Pérez-Silva et al., 1969). The elongation phase is preceded by a loss of DNA, possibly in the form of giant chromosomes (Ammermann, 1965, 1968) so it is unlikely that there is any gene activation prior to this stage.

To explain the rejuvenating effect of regeneration one must propose that a new pattern of gene activity is also initiated in the regenerating fragments. This could occur at about the same time when these fragments also begin to elongate; when in size, shape and staining intensity they resemble the differentiating anlagen of animals undergoing a complete autogamy.

Determination of gene activity during macronuclear reorganisation is not without precedent. For example, in P.aurelia a particular genotype can determine

more than one phenotype. In this species, as already mentioned the synkaryon divides twice, and two macronuclear anlagen develop, to be distributed to the daughters at the first fission. In syngen 1 although genotypically identical, these two cells can give rise to two caryonides which express different mating types. During the development of the two anlagen within a single animal, they may both become determined for mating type I, or both for mating type II, or one for mating type I, and the other for II. The nuclear differentiation achieved early in the life of the macronucleus is usually stable until the next autogamy or conjugation. In his review of caryonidal inheritance Preer (1969) quoted examples of this phenomenon in Paramecium and Tetrahymena. The evidence that cytoplasmic factors influence the future phenotype is provided by the fact that the type of cytoplasm in which the anlagen are developing is important.

The foregoing speculation implies that macronuclear regeneration in E.minuta can rejuvenate because a new cycle of gene activity is initiated rather late in the development of the new macronucleus. The influence of temperature upon caryonidal inheritance in P.aurelia (Sonneborn, 1939a) shows that the critical period for determination of the phenotype, and therefore probably also for the start of a new round of gene action, is during the second division of the synkaryon, or very early in anlage formation. It is therefore not surprising that macronuclear regeneration, which in P.aurelia does not usually begin until after the first fission following sexual reproduction, neither results in immaturity, nor in any changes in gene expression in this species. One would not expect macronuclear regeneration to have a rejuvenating effect in P.aurelia, and indeed Nobili (1960, 1961) has demonstrated that in terms of fissions and fission rate the rejuvenating effect of successive macronuclear regenerations is absent or very small unless selection is employed.

Evidence of sequential gene activity during the life cycle of ciliates is

also growing. Allen (1968) describes growth cycle variations in enzymes, particularly the esterase-1 isozymes of T.pyriformis, some appearing sooner than others during logarithmic growth. In P.bursaria there is a temporal sequence for the expression of the two mating type loci (Siegel and Cohen, 1963; Siegel, 1965). When clones are first initiated from exconjugants no mating type substance is produced; as the clone is expanded a single substance is made, and finally cells express both loci. The phenomena of maturity and, where applicable, the ability to undergo autogamy, are also clearly cyclical in Tetrahymena, Paramecium and Euplotes. The immaturity of exautogamous clones of E.minuta, whether the new macronucleus is derived by regeneration or by reorganisation of the products of the synkaryon, strongly suggests that during both processes a new cycle of sequential gene activation and repression is initiated.

Maturity will mark the end of the life cycle. If no fertilisation process occurs, senescence, brought about by undesirable gene activation or repression, may set in. In a sense this is an extension of the theories of Bütschli (1876) who believed that macronuclei wore out with use, and of Woodruff and Erdmann (1914) that it is the mixing of macronuclear material with the cytoplasm that has the rejuvenating effect. The chromosomal imbalance which Faure-Fremiet thought occurred with age can also be visualised in terms of genes which no longer act in concert, or in sequence, as a result of stable or non-adaptive gene repression or derepression.

Several other authors (Jennings, 1942; Sonneborn, 1954a; Siegel, 1961, 1965; Preer, 1969) have also expressed opinions that concentrations of cytoplasmic factors, which may themselves be gene products, influence phases of the life cycle of ciliates, and when produced in an uncontrolled way these may contribute to the ageing process.

Concluding remarks

Comparisons of the genetic systems working in *Paramecium*, *Tetrahymena* and *Euplotes* serve to illustrate the uniqueness of different protozoa. Not only do taxonomic orders differ from one another in the type of sexual processes which we can observe, and in the stability of phenotypes, but considerable diversity is evident within a genus. Studies of *Euplotes minuta* now show that a single gene controlling the ability to undergo autogamy may produce such variation within a species.

This study has established that the macronuclei of exautogamous *E.minuta* are usually derived by regeneration. Hence a change in genotype, and therefore phenotype, is rare following autogamy. The origin of the micronucleus in regenerate clones is still unknown. There is a need for a further genetic study of the micronuclear genotype using loci other than the mating type locus. Use of radioactive isotopes and autoradiography might help to solve the problem of how the micronucleus is formed, and would enable regeneration of the macronucleus to be studied by an independent technique.

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